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SECURIN-RELATED CELL PROLIFERATION IN BREAST CANCER PROGNOSIS

Heli Repo

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To my family

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ABSTRACT

Breast cancer is the most common malignancy among women. It remains a major cause of mortality, although the five-year survival of breast cancer patients is about 90%. To aid in the clinical treatment decisions, clinical factors and biomarkers are utilized to predict the behaviour and prognosis of breast cancer. Traditionally such prognostic factors have been the size and stage of the tumour, hormone receptor expression, the amplification status of *Her2* oncogene and the proliferation activity of the tumour cells.

The purpose of this study is to investigate regulatory proteins of the metaphase-anaphase transition of the cell division and evaluate their potential value in predicting the prognosis of breast carcinoma patients. The study is based on 1135 breast cancer patients with a maximum follow-up time of 22 years. The tissue material was collected into tissue microarrays and the protein expressions were analysed with immunohistochemical and immunofluorescence methods.

Securin, PTTG1IP, separase and SA2 are proteins involved in the regulation of the cell cycle. In this study, the immunohistochemical expression of these proteins in breast cancer tissue was examined. The changes in the expression profile were then used to estimate their prognostic value. Securin overexpression alone predicted a 2.4-fold risk of breast cancer death ($p>0.001$). Combined with the other studied cell-cycle proteins, this risk was emphasized. A model combining securin, separase and axillary lymph node status increased the risk of breast cancer death 6.2-fold (p 0.0006, CI 3.2-82.6). In addition, cytoplasmic securin expression was associated with triple negative subtype of breast cancer.

Based on this study securin-related cell cycle proteins are promising new candidates as biomarkers for breast cancer prognosis.

KEYWORDS: Breast cancer, prognosis, cell cycle, securin, PTTG1IP, separase, SA2

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Rintasyöpä on naisten yleisin syöpä. Viiden vuoden jälkeen sairastuneista naisista elossa on n. 90%. Hyvistä hoitotuloksista huolimatta rintasyöpään liittyy siis edelleen huomattavaa kuolleisuutta. Rintasyövän käyttäytymisen ennustaminen on perinteisesti perustunut kasvaimen kokoon, taudin levinneisyyteen, hormonireseptorien ja *Her2*-onkogeenin ilmentymiseen syöpäsoluissa sekä kasvainsolujen jakautumisaktiivisuuteen.

Tämän tutkimuksen tarkoituksena on selvittää uusia rintasyövän taudinkulkuun ja potilaiden ennusteeseen vaikuttavia tekijöitä. Tutkimus perustuu yhteensä 1135 rintasyöpäpotilaan aineistoon ja enimmillään 22 vuoden seuranta-aikaan. Potilaiden kudosnäytteistä valmistettiin monikudosblokit, joita tutkittiin immunohistokemiallisin ja immunofluoresenssi-menetelmin.

Sekuriini, PTTG1IP, separaasi ja SA2 ovat solunjakautumisen säätelyyn osallistuvia proteiineja. Tutkimuksessa määritettiin näiden proteiinien immunohistokemiallista ilmentymistä rintasyöpäkudoksessa sekä värjäytymisprofiilin muutosten vaikutusta potilaiden ennusteeseen. Sekuriinin yli-ilmentyminen yksinään ennusti 2.4-kertaista rintasyöpäkuoleman riskiä ($p>0.001$). Yhdistettynä muihin solunjakautumisen säätelyyn osallistuviin proteiineihin riski korostui. Sekuriini, separaasi ja kainaloimusolmukestatus ennustivat 6.2-kertaista riskiä (p 0.0006, CI 3.2-82.6). Sekuriinin ilmentyminen solun sytoplasmassa liittyi voimakkaasti huonoennusteiseen kolmoisnegatiiviseen rintasyöpään.

Tutkimuksen perusteella sekuriiniin liittyvät solunjakautumista säätelevät proteiinit ovat lupaavia tulevaisuuden biomarkkereita rintasyövän ennusteenarviointia ja yksilöllisiä hoitopäätöksiä varten.

AVAINSANAT: rintasyöpä, ennuste, solunjakautuminen, sekuriini, PTTG1IP, separaasi, SA2

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Abbreviations

APC/C	Anaphase-promoting complex/cyclosome
bFGF-2	Basic fibroblast growth factor
BUB1	Budding uninhibited by benzimidatsoles 1
BUBR1	Budding uninhibited by benzimidatsoles 1 -related protein kinase
BRCA	Breast cancer gene
CDC20	Cell division cycle 20
CDK	Cyclin-dependent kinase
CI	Confidence interval
CISH	Chromogenic *in situ* hybridization
DCIS	Ductal carcinoma in situ
ER	Oestrogen receptor
EGFR	Epidermal growth factor receptor
ESPL1	Extra spindle poles-like 1
H&E	Haematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
IHC	Immunohistochemistry
Mad2	Mitotic arrest deficient protein 2
mTOR	Mammalian target of rapamycin
NST	No special type
NLS	Nuclear location signal
OR	Odds ratio
PARP	Poly (ADP-ribose) polymerase
PBF	Pituitary tumour transforming gene binding protein
PD-L1	Programmed death -ligand 1
Plk-1	Polo-like kinase 1
PR	Progesterone receptor
PTTG1	Pituitary tumour transforming gene 1
PTTG1IP	Pituitary tumour transforming gene 1 interacting protein
Rad21	Double-strand-break repair protein 21
Rb	Retinoblastoma tumour suppressor

SA2	Stromal antigen 2
SAC	Spindle assembly checkpoint
SMC	Structural maintenance of chromosomes
STAG2	Stromal antigen 2
STK11	Serine/threonine kinase 11
TIL	Tumour infiltrating lymphocyte
TMA	Tissue microarray
TNBC	Triple-negative breast cancer
VEGF	Vascular endothelial growth factor

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Repo H, Löyttyniemi E, Nykänen M, Lintunen MM, Karra H, Söderström M, Kuopio T, Kronqvist P: Expression of cohesin subunit SA2 predicts breast cancer survival. *Appl Immunohistochem Mol Morphol*. 2016; 24:615–621.

II Gurvits N, Repo H, Löyttyniemi E, Nykänen M, Anttinen J, Kuopio T, Talvinen K, Kronqvist P: Prognostic implications of securin expression and sub-cellular localization in human breast cancer. *Cell Oncol (Dordr)*. 2016; 39:319–331.

III Repo H, Gurvits N, Löyttyniemi E, Nykänen M, Lintunen MM, Karra H, Kurki S, Kuopio T, Talvinen K, Söderström M, Kronqvist P: PTTG1-interacting protein (PTTG1IP/PBF) Predicts Breast Cancer Survival. *BMC Cancer*. 2017; 17: 705.

IV Repo H, Löyttyniemi E, Kurki S, Kallio L, Kuopio T, Talvinen K, Kronqvist P: A prognostic model based on cell-cycle control predicts outcome of breast cancer patients (Manuscript)

1 Introduction

Cancer progression is dependent on sustaining proliferative signalling, evading growth suppressors and the immune system, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg 2011). Loss of proliferative control is a common feature of cancer caused by cell cycle dysregulation (Williams and Stoeber 2012). The controlled maintenance, release and dismantling of sister chromatid cohesion has been described as one of the key elements in ensuring the normal progression of the cell cycle. The distribution of DNA during meta-phase/anaphase transition is one of the critical events of the cell cycle controlled by an active process involving a network of regulatory proteins, the function of many of these presently poorly understood. Mistakes in this process may lead to genetic instability, characterized by an increasing number of genetic alterations, the loss of cell cycle control and DNA repair, promoting tumorigenesis (Hanahan and Weinberg 2011).

Proliferation has been shown a strong indicator of disease progression and outcome in several malignancies, including breast cancer. Mitotic functions are also exploited in cancer treatment. Blocking mitotic exit has also been speculated as an efficient anticancer strategy. Disturbing the cellular functions during cell cycle may lead to abnormal microtubule assembly or spindle assembly and cause cell cycle arrest in cancer cells (Dominguez-Brauer et al 2015).

Cell division is a tightly controlled process, in which interactions between protein cascades manage the equal division of genetic material to the daughter cells. Errors in this process may lead to cell death but also to aneuploidy and an escape from checkpoint control (Giam and Rancati 2015, Potapova and Gorbsky 2017). The spindle assembly checkpoint delays the onset of meta-phase until all the chromatids have been properly aligned in anaphase. This delay is provided by a protein complex called anaphase promoting complex/cyclosome (APC/C). Once the chromatids are properly aligned, activation of APC/C leads to the destruction of securin, initiating the protein cascade and leading to the separation of sister chromatids.

Presently, clinical and histological features and a small set of molecular biomarkers are utilized to direct the management of breast cancer patients (Rakha and Ellis 2011, Goldhirsch et al 2013). Molecular techniques have shown potential

in research approach but their actual role in clinical practice is still limited (Goldhirsch et al 2013). Biology-driven research applied in immunohistochemical detection of biomarkers still provides a reliable, fast and inexpensive method for clinical use (Fumagalli et al 2012). The last decades have emphasized the need for accurate prognostic and predictive information for individualized treatment of breast cancer since molecular taxonomy has revealed the molecular heterogeneity of the disease (Januškevičienė and Petrikaitė 2019).

The present study focuses on immunohistochemical identification of novel, clinically applicable biomarkers, securin, PTTG1IP, separase, SA2, with potential to aid in personalized treatment decisions in breast cancer.

2 Review of the Literature

2.1 Clinical features of breast cancer

2.1.1 Incidence

Breast cancer is the most common malignancy and cause of cancer deaths among women in both Finland and worldwide. According to Finnish Cancer Registry every 8th Finnish woman will be diagnosed with breast cancer during her lifespan and the incidence has more than tripled during the last 50 years (Finnish Cancer Registry 2019). In 2017, 4947 new cases of breast cancer were diagnosed in Finland, comprising one third of all malignancies among women. In the same year, 923 women died of breast cancer accounting for approximately 15% of all female cancer deaths. Worldwide, breast cancer comprises 12% of new malignancies among women (Ferlay J et al 2018).

2.1.2 Etiology

Breast cancer is a multifactorial disease (Lakhani et al 2012). The development of breast cancer is hormone-dependent and associated with life-long exposure to oestrogen. Thus, a substantial risk factor is the exposure to both endogenous and exogenous sex hormones, including early menarche, nullipara, late pregnancies and menopause (Barlow et al 2006). Recent and long-term menopausal hormone replacement therapy also increases the risk of breast cancer, especially when combined with progestin (Collaborative Group on Hormonal Factors in Breast Cancer 2019). Risk of breast cancer increases with age (Barlow et al 2006). Body mass index has been shown to associate with the risk of breast cancer, especially among postmenopausal women (Luo et al 2019). Lifestyle features associated with obesity, i.e. the lack of physical activity and high energy nutrition (Chlebowski 2013), as well as alcohol abuse (Seitz et al 2012) and smoking (Gaudet et al 2017) have also been presented as risk factors for breast cancer.

There are also known genetic risk factors for breast cancer. The most common dominant mutations are in the DNA repair genes BRCA1 and BRCA2. The incidence of inactivating, clinically relevant BRCA-mutations in Europe is 0.12–0.32% and,

in addition to breast cancer, they are also associated with pre-disposition to other malignancies, such as ovarian or fallopian carcinomas (Lakhani et al. 2012, Paul and Paul 2014). By 70 years of age, carriers of a mutated BRCA1 gene have a 65% risk and carriers of a mutated BRCA2 gene a 45% risk of developing breast cancer (Paul and Paul 2014). In addition, increased risk of breast cancer may be associated with other syndromes with generally increased risk of malignancies such as Li-Fraumeni syndrome (TP53) or Cowden's syndrome (PTEN) (Paluch-Shimon et al 2016).

2.1.3 Classification

Invasive breast cancer is not a single entity. It can be divided into several different histological entities based on the morphology and the molecular profile of the tumour (Lakhani et al. 2012). The most common subtype is called invasive ductal or carcinoma of no special type (carcinoma NST) (Fig. 1). This subtype comprises 40-70% of all breast cancer cases. The second most common subtype (5–15% of breast cancer cases) is invasive lobular breast cancer, characterized by a growth pattern with strand-like infiltration of cancer cells caused by the loss of E-cadherin expression. The remaining cases represent rare subtypes, e.g. tubular, mucinous or papillary carcinomas, each comprising 1–2% of all breast carcinomas. The morphological features of the carcinomas are routinely scored from haematoxylin and eosin (H&E) - stained histological specimen and combined into histological grades I–III (Figure 1).

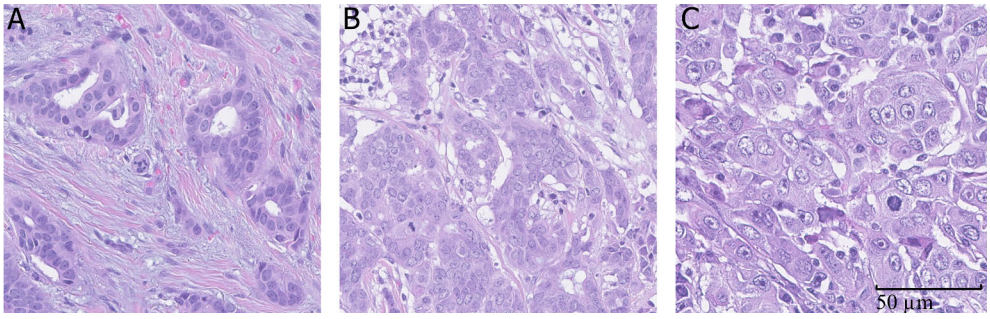

Figure 1 Microphotographs representing morphology of ductal breast carcinoma comprising infiltration of cancer cells in tubular structures. Low (grade I) (A), moderate (II) (B), and high (III) (C) histological grades are characterized by the extent of tubule formation, nuclear atypia and mitotic activity.

2.1.4 Molecular subtyping of breast cancer

In addition to the traditional histological subgroups, breast cancer is classified according to molecular expression patterns established based on gene-expression experiments (Perou et al 2000, Sørlie et al 2001, van 't Veer, L J et al 2002). The

most established division is into the intrinsic subtypes luminal A, luminal B, human epidermal growth factor 2 (HER2) –enriched and basal-like carcinomas. As the gene expression –based classification can be expensive and time consuming in clinical practice, the St Gallen International Breast Cancer Conference Expert Panel has presented surrogate markers, which may be used for the same purpose (Goldhirsch et al 2011, Coates et al 2015) (Table 1).

Table 1 Summary of surrogate markers for intrinsic classification of breast carcinomas.

Subtype	Criteria
Luminal A	*ER+, PR+, *HER2* -, Ki-67 < 20-29%
Luminal B (*HER2*-negative)	ER+, *HER2* -, Ki-67 > 20-29% or PR < 10%
Luminal B (*HER2*-positive)	ER+, *HER2*+, any Ki-67, any PR
HER2-positive	*HER2*+, ER-, PR-
Triple-negative	ER-, PR-, *HER2*-

*Abbreviations used in this table: ER oestrogen receptor, PR progesterone receptor, *HER2* human epidermal growth factor 2, Ki-67 proliferation marker

2.1.5 Clinical prognostic and predictive markers of breast cancer

The histological diagnosis of breast cancer contains only a limited amount of information on the progression and the prognosis of the disease. For informed decisions on treatment of an individual patient additional information is required. For this purpose, there are several well established clinical, histopathological and molecular prognostic and predictive markers.

Significant prognostic information on the expected course of the disease can be obtained from the clinical prognostic features. These include tumour size, axillary lymph node status and the presence of distant metastases. These features are evaluated individually and combined into the staging of the disease in the tumour, lymph node and metastasis (TNM) classification (Senkus et al 2015, Cserni et al 2018).

The histopathological characteristics providing evidence on the behaviour of the disease include the extents of tubule formation, nuclear atypia and mitotic activity (Fig. 1). Other morphological features, such as the histological type, specific growth pattern, cellular differentiation, the extent of *in situ* –component and presence of lympho-vascular invasion are also taken into consideration in treatment decisions (Senkus et al 2015).

Prognostic and predictive biomarkers of breast cancer include immunohistochemical detection of oestrogen (ER) and progesterone (PR) receptors, *HER2* amplification status and the extent of cell proliferation evaluated using e.g. Ki-67 –antibody. After screening with IHC, *HER2* amplification is further verified using *in situ* hybridization (Wolff et al 2013). These results are used as the surrogate criteria for the intrinsic classification as outlined in chapter 2.1.4.

Recently, markers of the tumour microenvironment, tumour stroma and inflammatory cells have been added to the analysis of breast cancer development and prognosis. For instance, analysis on the nature of tumour infiltrating lymphocytes (TILs) (Loi et al 2014) and the expression of the programmed death -ligand 1 (PD-L1) (Bertucci and Gonçalves 2017) have shown to work as tools for the prognostic evaluation of breast cancer.

2.1.6 Clinical management of breast cancer

The treatment decisions of an individual patient are based on so called triple-diagnostics involving clinical, radiological and histopathological information. Treatment decisions are based on international and national guidelines, provided by expert panels (Table 2). Surgery is still the gold standard of treatment of localized breast cancer, although it is increasingly preceded by neoadjuvant treatments. After primary surgery the decision of adjuvant therapy involving radiation or cytotoxic systemic therapies is made. The management of metastatic disease is often still challenging. Recent discoveries such as immunotherapy targeting specific molecular pathways and combinations of chemotherapy and biological treatments are providing new tools for the treatment of these patients (Senkus et al 2015, Curigliano et al 2017). Particularly, in the aggressive triple-negative subgroup anti-PD-L1 treatments are showing promising results (Lazarus et al 2019).

Table 2 The adjuvant treatment of breast cancer after primary surgery.

Low risk of recurrence	
Tumour size <10mm, lymph node negative, ER+, PR+, *HER2* -	Either no adjuvant therapy or endocrine therapy
Intermediate risk of recurrence	
Tumour size 11–20mm, lymph node negative, grade I–II ER+, PR+, *HER2*-	Endocrine therapy
Tumour size 11–20mm, lymph node negative, grade II–III ER+, PR+, HER2-	Chemotherapy and endocrine therapy
High risk of recurrence	
Age <35yrs, Tumour size >20mm, lymph node positive, all triple-negative*, all HER2+*	Chemotherapy and endocrine therapy if ER ≥ 1%
	HER2+: chemotherapy and trastuzumab

Modified from the Finnish Breast Cancer Group 2019 guidelines

* apart from tumour size <5mm, lymph node negative

2.2 Cell proliferation

2.2.1 The cell cycle

The maintenance of life is mainly owed to the controlled maintenance of chromosomal integrity during the cell division of the cell cycle. Both the genetic

information in chromosomes and the cell organelles are doubled during the cell cycle to ensure an identical copy or the original cell.

During the cell cycle, the chromosomes are duplicated at the S-phase. The cell division the occurs during the M-phase. Since the eukaryotic cells require time to transition between these two active phases, there are two gap phases in the cell cycle (Figure 2). The first gap (G1) phase takes place at the beginning of each cell cycle, after M phase has finished. The second gap (G2) happens between S phase and M phase, allowing the cell time to prepare for cytokinesis at the end of M phase. The gap periods also allow the cell to perform the necessary checkpoint control to assure that the cell is completely prepared for correct division for safe progression to the next phase. (Malumbres and Barbacid 2009, McIntosh et al 2012)

Not all cells take part in the cell cycle. Some cells, such as neurons, are in a permanently and irreversibly located in G0 state. In some cells, the G0 state is transient and the cell returns to the cycle dependent on specific signalling, e.g. phosphorylation in case of retinoblastoma protein. (Ren and Rollins 2004)

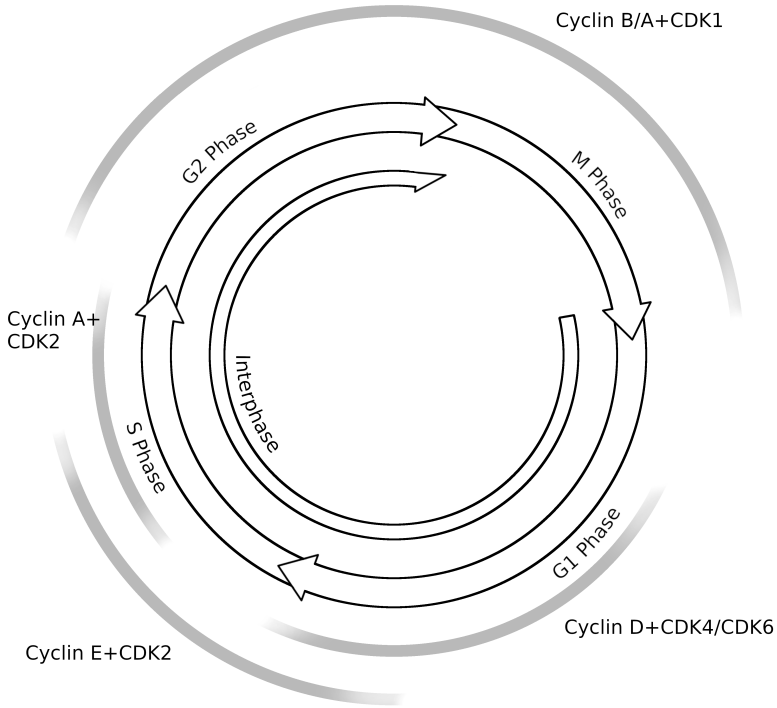

Figure 2 The cell cycle consists of four phases named G1 (gap 1), S (synthesis), G2 (gap 2) and M (mitosis). The transition between phases is controlled by cyclin-dependent kinases (CDKs).

2.2.2 The control of the cell cycle

The control of the cell cycle is performed by a complex network of protein interactions and feedback signalling. The feedback signals function as biochemical switches, triggering or inhibiting the surrounding processes. The main regulators of the cell cycle are the interactions between cyclins, cyclin-dependent kinases (CDK) and CDK-inhibitors, proteins synthetized and degraded in a highly stable manner at different phases of the cell cycle. (Malumbres and Barbacid 2009)

The progression of the cell cycle requires that cyclins are activated by forming complexes with CDKs (Fig. 2). The active cyclins can be divided into four different classes, named after the period of the cell cycle during which they are active. Thus, the G1, G1/S, S and M cyclins are active during the G1, late G1, S and M phases, respectively. The full activity of the cyclin-CDK –complex is only achieved after phosphorylation by a CDK-dependent kinase. (Malumbres and Barbacid 2009) In addition to cyclins, specific inhibitory proteins are known to control the cell cycle progression (Besson et al 2008).

2.2.3 Mitosis

The mitoses of the cell cycle can be divided into five stages: prophase, prometaphase, metaphase, anaphase and telophase (Figure 3).

At prophase, DNA replication is complete, and the sister chromatids are held together by catenation of the DNA and cohesin rings. The cohesin ring is multiprotein complex, consisting of four different subunits. In mammalian cells these proteins are structural maintenance of chromosomes proteins 1 and 3 (SMC1, SMC3), double-strand-break repair protein 21 (Rad21) and stromal antigens 1 or 2 (SA1/SA2) (Losada 2014). During prophase most of cohesin encircling the sister chromatid arms is removed by a process controlled by Aurora B and polo-like kinase 1 (Plk-1) in a phosphorylation related manner (Gimenez-Abian et al 2004). The cohesin at the centromeres is protected from removal by a shugosin (McGuinness et al 2005).

Further, during prophase the duplicated centrosomes move to opposite poles and begin to form the mitotic spindle, still leaving the nuclear envelope intact. At prometaphase, the nuclear membrane is dissolved and the sister chromatids, attached by cohesin at the centromeres and loosely at the chromatid arms, begin to assemble to the equator of the cell, guided by the microtubules of the mitotic spindle. At metaphase the sister chromatids are aligned between the centrosomes, attached from the kinetochores by the microtubules. (McIntosh et al 2012)

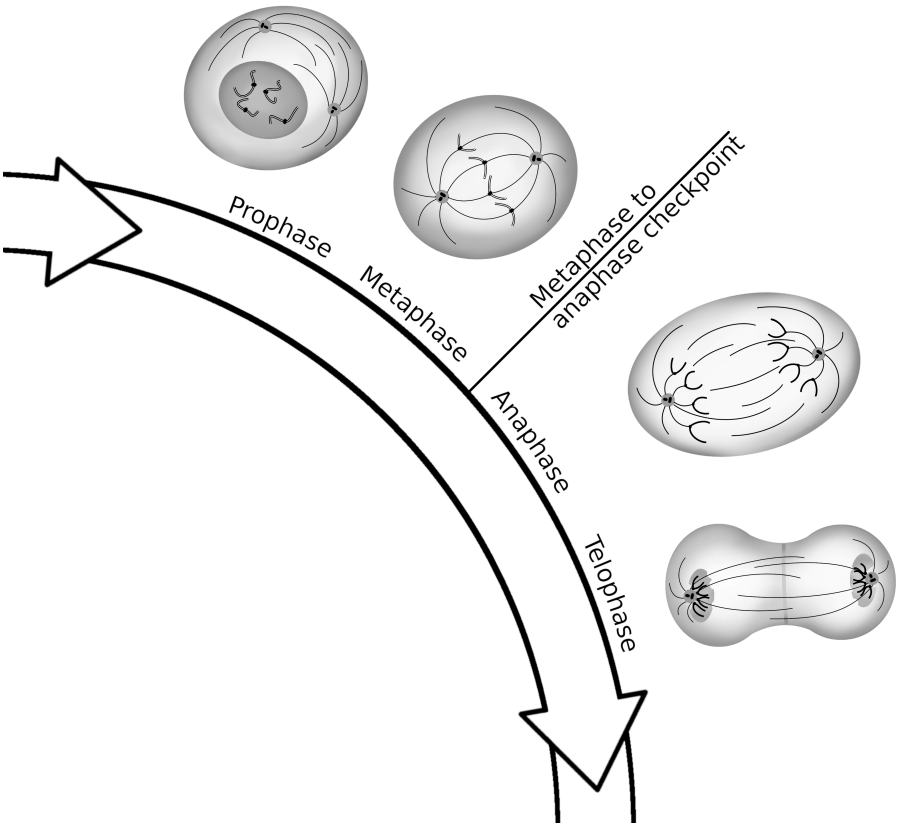

Figure 3 Mitosis consists of five phases, each with distinct events leading to formation of identical daughter cells. Prometaphase is not illustrated in this image.

The metaphase to anaphase transition is a point of no return. The cohesion be-tween sister chromatids is dissolved allowing the chromosomes to be pulled by the spindle microtubules towards the opposite poles (McIntosh et al 2012). This event depends on the cell passing quality control at the spindle assembly checkpoint (SAC). A key switch for this is the activation of anaphase-promoting complex/cyclosome (APC/C) by cell division cycle protein 20 (CDC20) (Lara-Gonzalez et al 2012, Musacchio 2015). Activation of this complex leads to the degeneration of cyclin B and the inactivation of CDK1 initiating sister chromatid separation (Musacchio 2015).

Before anaphase, the sister chromatids are bound together by the remains of the cohesin rings. Separase, the protein required for the removal of cohesin rings are kept inactive by securin, preventing the cleavage of cohesin and the premature separation of the sister chromatids (Ciosk et al 1998) (Figure 4). At SAC, the activation of APC/C leads to ubiquitinylation and, eventually, the degradation of securin (Shirayama et al 1999), releasing the activated separase. The actual release

of the sister chromatids occurs, when separase in turn cleaves the Rad21 portion of cohesin from both the centromeres and the residual from chromosome arms (Uhlmann et al 1999, Waizenegger et al 2000, Hauf et al 2001, Gimenez-Abian et al 2004), releasing the sister chromatids. Cohesin cleavage is essential for the normal progression of mitosis (Hauf et al 2001). At the same time, release of securin from separase leads to autocatalytic destruction of separase (Waizenegger et al 2000).

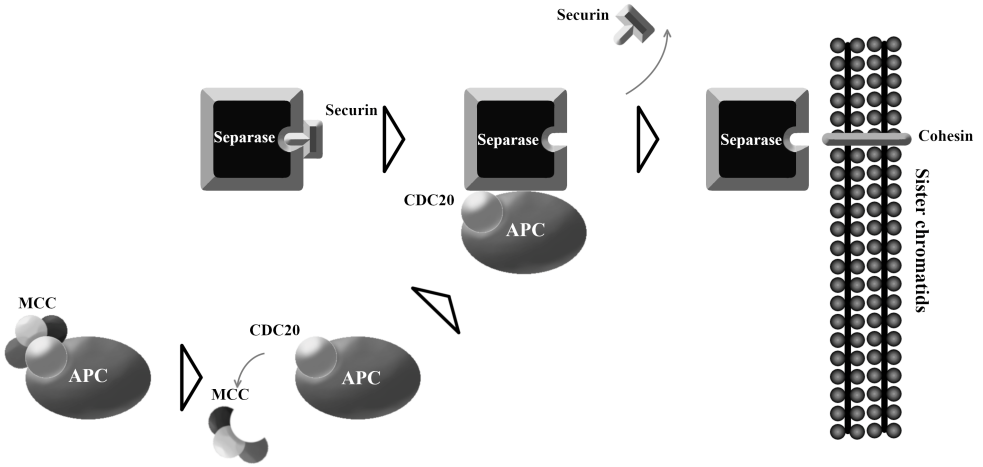

Figure 4 Transition from metaphase to anaphase involves the sequential activation and inactivation of APC/C, CDC20, securin, separase and cohesin.

At the end of anaphase, the separated sister chromatids are then pulled to the opposite sides of the mitotic spindle by the tension caused by the microtubules (McIntosh et al 2012). At telophase the separated chromosomes arrive at the poles of the mitotic spindle and decondense and the nuclear envelope begins to reconstruct. Finally, at cytokinesis the cytoplasm and the cell organelles are divided into two daughter cells, both with a single nucleus (Pollard 2009).

2.2.4 Cell cycle checkpoints

Maintaining the accuracy of the DNA replication and the proper alignment and separation of the sister chromatids is ensured by specific checkpoints. Each checkpoint serves as a pathway where the cell cycle progression may be halted in case the specific conditions of the cell cycle are not met. The checkpoints are named after their location during the cell cycle as G1, G2/M and the spindle assembly checkpoint (SAC).

2.2.4.1 G1 (restriction) checkpoint

The G1 checkpoint prevents the cell from initiating the cell cycle unless the surrounding environment is favourable. This occurs as a two-step process, the first step taking place early in G1 ensuring that enough growth factor signalling is present. This checkpoint is controlled by the phosphorylation of the retinoblastoma tumour suppressor (Rb) and is mediated by the G1 cyclins. The second step takes place in late G1, close to S phase, regulating that the nutritional requirements are present. A focal point for this is the function of mammalian target of rapamycin (mTOR), with additional guidance from growth factors such as insulin, insulin like growth factor 1 and platelet derived growth factor. If the requirements are not met, a normal cell will enter quiescence (G0) instead of dividing. (Foster et al 2010)

2.2.4.2 G2/M transition checkpoint

The G2/M checkpoint prevents the diving cell from progressing into mitosis before identifying and repairing defected DNA after replication. If this checkpoint is defective, G2/M transition occurs despite DNA damage leading to apoptosis and cell death. The activity of the cyclinB-CDK1 complex is pivotal in regulating the G2-phase transition wherein CDK1 is maintained in the inactive phase. (Malumbres and Barbacid 2009)

2.2.4.3 Spindle assembly checkpoint

The spindle assembly checkpoint (SAC) takes place at the metaphase-anaphase transition with the purpose to prevent premature sister chromatic separation. A single misaligned chromosome or unattached microtubule is enough to trigger the SAC and halt cell cycle progression until all the chromosomes are properly aligned (Rieder et al 1995). The checkpoint proteins are located at the kinetochore (Rieder et al 1995) and the function of the checkpoint is partly mediated by the tension at the kinetochore (Shannon et al 2002).

Whereas the G1 and G2/M checkpoints are based on the cyclin-CDK interactions and protein phosphorylation, SAC is driven by protein destruction (Lara-Gonzalez et al 2012). The gatekeeper of this process in the anaphase promoting complex/cyclosome (APC/C), a member of the ubiquitin ligase enzymes. The APC/C functions as a catalyst, initiating ubiquitinylation and thus the destruction of other proteins (Lara-Gonzalez et al 2012). This enzyme function is activated by the binding of CDC20 to APC/C, creating the APC/C^{CDC20} complex (Lara-Gonzalez et al 2012, Musacchio 2015). This event also involves complex regulation by additional proteins, such as mitotic arrest deficiency 2 (Mad2), budding uninhibited by

benzimidatsoles 1 (Bub1) and Aurora kinase B, which are also involved in the activation (Musacchio 2015).

Once all the chromosomes are properly aligned and the mitotic spindle is properly attached, the amount of Mad2 at the kinetochores is radically reduced (Niault et al 2007). Activation of the APC/C^{CDC20} complex triggers cyclin B and securin in initiating the mitotic exit (Shirayama et al 1999, Musacchio 2015). Ubiquitinylation and degradation of securin, in turn, releases separase which initiates the dissociation of sister chromatids by cleaving the cohesin complex (Luo and Tong 2018).

2.2.5 Errors in mitosis

If the control for the cell cycle is disrupted, either by delays in the cell division or defects in the cell cycle checkpoints, missegregation of chromosomes may occur (Potapova and Gorbsky 2017). In missegregation there is a possibility that the chromosomal material is only doubled but not distributed into separate daughter cells or the distribution is otherwise unequal resulting in an abnormal chromosome number. The consequences to the cell vary from insignificant to catastrophic, depending on the nature and degree of the genetic error and the role of the cell in the organism (Giam and Rancati 2015). In normal cells, a p53-dependent surveillance system is responsible for detecting the abnormal chromosome content and halting the cell cycle and causing cell death or senescence (Thompson and Compton 2010). In malignancy, however, the p53 is often defective, halting this protective process.

Errors in the mitotic process predispose cells to the loss of tumour-suppressor genes, chromosomal instability, aneuploidy and carcinogenesis. However, the effects of aneuploidy may be complex and have both pro- and anti-cancerous effects. More than 60% of cancers exhibit aneuploidy with variation between tumour types and locations (Duijf et al 2013). Chromosomal instability is the cause for different subclones of cancer cells in a single tumour i.e. intratumour heterogeneity (Andor et al 2017). The accumulating genomic alterations may also give rise to tumour specific antigens, neoantigens, which are highly immunogenic and can be targeted by immunotherapy (Desrichard et al 2016).

A low level of chromosomal instability may provide an evolutionary edge but eventually the disarray may prove lethal for the cell (Andor et al 2017). Thus, intermediate levels of aneuploidy may be associated with a poor prognosis, but when taken to extreme levels the result may be adverse (Andor et al 2017). This may be exploited in cancer treatment. The DNA-damaging agents, such as cisplatin or DNA damage repair inhibitors, such as PARP (Poly(ADP-ribose)polymerase) inhibitors cause cell death by inducing DNA damage and inhibiting the repair processes, leading to an unsustainable level of chromosomal damage (Oliver et al 2010,

Livraghi and Garber 2015) Causes for the increased mitotic infidelity in chromosomal instability and aneuploidy include impaired sister chromatid cohesion, issues with mitotic timing, errors in the formation of the mitotic spindle or faulty double-strand break repair (Thompson et al 2010). One significant source of aneuploidy in tumour cells is delay or arrest at metaphase. Normally cells are halted in metaphase for only a few minutes, followed by the rapid onset of anaphase. In cancer, anaphase onset may be delayed at the metaphase-anaphase checkpoint because of the failure of one or more chromosomes to align in a timely manner. In the clinical setting, this phenomenon is being applied in antimitotic chemotherapy drugs targeting genomic instability (Yamada and Gorbsky 2006). Microtubule inhibitors, such as taxans, are used to induce mitotic arrest and prodding the cancer cells towards cell death instead of mitosis (Yamada and Gorbsky 2006).

2.3 Review of securin, PTTG1IP, separase and SA2

2.3.1 Securin

2.3.1.1 Securin/PTTG1 in normal cells

Securin, the protein product of the pituitary tumour-transforming gene 1 (*Pttg1*) was originally characterized in rat pituitary tumour cell line as a novel protein capable of transforming normal rat pituitary cells into tumour cells (Pei and Melmed 1997). Almost simultaneously the human homologue of securin was then cloned from human foetal liver (Zhang et al 1999) and Jurkat cells (Dominguez et al 1998). High securin expression was also detected in human adult testis and thymus (Dominguez et al 1998, Zhang et al 1999) and placenta (Dominguez et al 1998). In several other tissues such as colon, small intestine, brain, lung, pancreas, ovary, peripheral blood leucocytes and foetal liver the expression of *PTTG1* was found to be low or undetectable (Dominguez et al 1998, Zhang et al 1999). Even in the highly securin-expressing normal tissues, the expression levels were generally still lower than in carcinoma cell lines from both solid tumours and hematopoietic neoplasia (Dominguez et al 1998, Zhang et al 1999). The tumour transforming properties of *PTTG1* have also described both *in vitro* and *in vivo* in a mouse xenograft model (Dominguez et al 1998, Zhang et al 1999).

Securin expression was originally associated with mitosis and the cell cycle when identified as a substrate for APC/C and detected to be co-immunoprecipitated with the known cell cycle control protein separase (Zou et al 1999). The expression

of securin rises in the cell during the onset of S phase and rapidly declines after a peak in the G_2-M phases (Zou et al 1999).

It has been speculated that the subcellular localization is critical for the cellular function of securin. Securin is required in the nucleus to prevent the premature activation on separase (Ciosk et al 1998). Nuclear proteins are synthesized in cytoplasm and transported to nucleus via the nuclear pore complex. Typically, this transport requires nuclear localization signal (NLS). Securin lacks a nuclear location signal but its relatively small 22kDa size, should allow diffusion across the nuclear pore complex (Chien and Pei 2000). However, a binding factor of securin, pituitary tumour-transforming gene 1 interacting protein (PTTG1IP), has been found to participate in the nuclearization of securin (Chien and Pei 2000, Vlotides et al 2007).

In human tissues, securin has most commonly been reported as both cytoplasmic and nuclear (Dominguez et al 1998) in benign tissues, such as normal endometrium (Kim et al 2008) as well as in malignant cells of e.g. gliomas (Salehi et al 2013) and gastric carcinomas (Xu et al 2016). In normal oesophageal squamous epithelium securin has been described nuclear and expressing mainly in the proliferating cell layer (Ito et al 2008, Zhang et al 2014). It has been suggested that cytoplasmic securin may be associated with Golgi apparatus and have a role in microtubule nucleation (Moreno-Mateos et al 2011).

According to literature, in vertebrates securin is not an essential element in the cell cycle as some vertebrate cells lacking the *Pttg1* gene can proceed through the mitotic process (Mei et al 2001). Also, a *Pttg1* null/null mouse model is viable (Mei et al 2001, Wang et al 2001) and fertile (Wang et al 2001), although the mice show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia and abnormal cytological and chromosomal patterns (Wang et al 2001). The mice are also susceptible for the development of diabetes (Wang et al 2003) induced by beta-cell apoptosis (Chesnokova et al 2009). The bone marrow stem cells from *Pttg1* null mice show normal differentiation patterns but exhibit lower proliferation, increased senescence and increased expression of DNA repair genes (Rubinek et al 2007). Transgenic mice overexpressing *Pttg1* have slightly increased risk for the development of ovarian and fallopian carcinoma and papillary serous adenocarcinoma of the pancreas (Fong et al 2012). However, crossbreeding a *Pttg1* overexpressing transgenic mice with *Tp53*-mutated mice lead to an increased tumour development compared to either of the mutations alone suggesting that an additional factor is needed for the oncogenic properties of *Pttg1* (Fong et al 2012).

In the early studies, based on *in vivo* and *in vitro* experiments, the expression of *PTTG1* has been suggested to be ER-dependent in pituitary tumours (Heaney et al 1999). Consequently, overexpression of securin has been detected in ER-responsive rat pituitary tumour cell line (Fujimoto et al 1999). Also, exogenous administration of ER in mice models has been shown to increase the expression of *PTTG1* mRNA

and cause hypoplasia and tumorigenesis in pituitary cells (Heaney et al 1999). However, the levels of securin expression have not been shown to be controlled by ER *in vivo* (Fujimoto et al 1999). Weather this remains true for breast cancer has not been established.

In addition to its function in the cell cycle, securin also functions as a transcriptional factor by directly binding to DNA or by interacting with other proteins (Tong and Eigler 2009). By these means, securin has roles in several different cell functions. It has been suggested to promote epithelial-mesenchymal transition (Xiea and Wangb 2016). It has been reported to promote angiogenesis by upregulating the basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Ishikawa et al 2001, McCabe et al 2002). Moreover, it has been suggested to inhibit DNA double-strand break repair by disturbing the Ku heterodimer formation (Kim et al 2007). Securin induces p53 expression and apoptosis by binding to p53 and interfering with the DNA binding properties of the protein (Bernal et al 2002). Other transcriptional targets of securin also include *c-myc* (Pei 2001), p21 (Chesnokova et al 2005) and prolactin (Horwitz et al 2003). It has also been observed that depleting securin from the cells disturbs normal cell migration (Moreno-Mateos et al 2011).

2.3.1.2 Securin in cancer

From early on securin was found to be overexpressed in human malignancies (Dominguez et al 1998, Zhang et al 1999). After the discovery of the integral function of securin in the chromosomal separation, it was speculated that the tumour formation might be associated with defects in the sister chromatid separation and, thus, the development of aneuploidy (Zou et al 1999).

PTTG1 overexpression has also been detected in several other types of malignancies based on mRNA and protein expressions. Table 3 presents an overview of different malignancies where this overexpression has been described in the literature.

Concerning prognostic implications, the overexpression of securin is also reported to predict adverse outcome in several malignancies. These prognostic implications are outlined in Table 4.

In literature, the overexpression of securin has been associated with a higher stage (Heaney et al 2000, Kim et al 2007, Cui et al 2015), an increased rate of lymph node invasion (Rehfeld et al 2006, Ito et al 2008, Yan et al 2009, Zhang et al 2014) and an increased rate of tumour recurrence (Solbach et al 2004, Solbach et al 2006, Filippella et al 2006, Raverot et al 2010) in several different malignancies.

In breast cancer, securin has widely been reported as overexpressed both immunohistochemically and on gene expression level (Puri et al 2001, Solbach et al

2004, Ogbagabriel et al 2005, Yoon et al 2012). This has also been confirmed in our own studies, where a cDNA microarray analyses involving 4000 cancer-related genes and a comparison of gene expressions between specimen of human breast carcinomas and non-cancerous breast tissue revealed *PTTG1* as the most differentially expressed (Talvinen et al 2008). In further studies, high securin expression in cancer cells was associated with aneuploidy and an increased risk of breast cancer related death (Talvinen et al 2009, Karra et al 2012).

Overexpressed securin in breast cancer tissue has been associated with an increased risk of lymph node invasion (Solbach et al 2004, Ogbagabriel et al 2005, Grizzi et al 2013) and risk of a relapse after treatment (Solbach et al 2004, Ghayad et al 2009). It has been suggested that this might be mediated by epithelial-mesenchymal transition (Yoon et al 2012). Overexpressing securin expression was found to lead to the acquisition of invasive properties in breast cancer cell lines (Yoon et al 2012). This has raised securin as a potential candidate for treatment, as depleting securin from cells decreased cell proliferation in breast cancer cell lines (Xiea and Wangb 2016) and suppressed tumour growth (Yoon et al 2012). Also, depleting securin from breast cancer cells appears to enhance radiosensitivity and induces senescence in the cancer cells, possibly due to disruption of the DNA double-strand break repair function of securin (Chen et al 2010).

Table 3 Examples of literature on the overexpression of securin in human malignancies.

Malignancy	Source	Material	Publication
Adrenocortical cancer	Protein	Tissue, cell lines	Demeure et al 2013, Romero Arenas et al 2018
Breast cancer	Protein, mRNA	Tissue, cell lines	Puri et al 2001, Solbach et al 2004, Ogbagabriel et al 2005, Ghayad et al 2009, Yoon et al 2012, Grizzi et al 2013
Cervical cancer	mRNA	Tissue	Guo et al 2019
Clear cell renal cancer	Protein, mRNA	Tissue	Wei et al. 2015
Colorectal cancer	mRNA, Protein	Tissue, cell lines	Heaney et al 2000, Kim et al 2007, Ren and Jin 2017
Gastric carcinoma	Protein, mRNA	Tissue	Wen et al 2004, Xu et al 2016
Head and neck squamous cell carcinoma	mRNA, Protein	Tissue	Solbach et al 2006, Heikkinen et al 2016, Ma et al 2018
Haematological neoplasia	Protein	Tissue	Dominguez et al 1998, Saez et al 2002
Hepatocellular carcinoma	Protein, mRNA	Tissue, cell lines	Cho-Rok et al 2006, Fujii et al 2006
Lung cancer	Protein, mRNA	Tissue, cell lines	Rehfeld et al 2006, Kakar 2006
Malignant gliomas	Protein, mRNA	Tissue, cell lines	Genkai et al 2006, Salehi et al 2013, Cui et al 2015
Medullary thyroid cancer	Protein	Tissue	Pezzani et al 2019
Mucoepidermoid carcinoma of the salivary gland	Protein, mRNA	Tissue, cell lines	Liu et al 2015
Multiple myeloma	Protein, mRNA	Blood, cell lines	Chiriva-Internati et al 2008
Oesophageal squamous cell carcinoma	Protein, mRNA	Tissue, cell lines	Zhou et al 2005, Ito et al 2008, Yan et al 2009, Zhang et al 2014
Ovarian cancer	Protein, mRNA	Tissue, cell lines	Puri et al 2001, El-Naggar et al 2007, Wang et al 2015
Pancreatic ductal carcinoma	Protein, mRNA	Tissue, cell lines	Grutzmann, Pilarsky et al 2004, Lin, Chen et al 2013
Prostate cancer	Protein	Tissue, cell lines	Zhu et al 2006, Cao et al 2012
Testicular tumours	mRNA	Tissue	Puri et al 2001, Pierconti et al 2015
Thyroid carcinoma	Protein, mRNA	Tissue	Boelaert et al 2003

Table 4 Summary of prognostic implications of securin in different malignancies reported in the literature.

Malignancy	Method	Cohort (n)	Over-expressing Fraction (%)	Subcellular location in IHC	Prognostic implications	Publication
Adrenocortical carcinoma	qRT-PCR	44	NA		↓ median survival 1.8 years with high expression vs. 9.0 years with low expression	Demeure et al 2013
Clear cell renal cell carcinoma	IHC qRT-PCR	192 44	58.9	nuclear and cytoplasmic	↓ mean survival 59.72 months with high expression vs. 88.28 months with low expression	Wei et al 2015
Colorectal carcinoma	IHC	118	NA		↓	Ren and Jin 2017
Gastric cancer	IHC qRT-PCR	78 98	74.4	cytoplasmic and nuclear	↓ mean survival 34.8 months with high expression vs. 62.6 months with low expression	Xu et al 2016
Hepatocellular carcinoma	IHC qRT-PCR	62	NA		↓ overall survival rate 58.6 % with high expression vs. 82.3 % with low expression	Fujii et al 2006
Laryngeal squamous cell carcinoma	IHC	210	88	cytoplasmic and nuclear	↓ mean survival 28.1 months with high expression vs. 38 months with low expression	Ma et al 2018
Non-small cell lung carcinoma	IHC	91	97.8		↓ mean survival 306 days with high expression vs. 463 days with low expression	Rehfeld et al 2006
Oesophageal squamous cell carcinoma	IHC	108/113	38.0/60.2		↓	Zhang et al 2014, Ito et al 2008
Skin squamous cell carcinoma	IHC	26	96	nuclear and cytoplasmic	expression levels showed no prognostic value	Ishitsuka et al 2013
Small cell lung carcinoma	IHC	136	64		↑ mean survival 379 days with high expression vs. 265 days with low expression	Rehfeld et al 2006

↓ outcome, ↑ outcome NA= not reported IHC= immunohistochemistry qRT-PCR: real-time reverse transcription-PCR

2.3.1.3 Subcellular location of securin in cancer

In the literature, research on the subcellular location of securin in cancer are sparse and reports variable observations between in different research settings and in different cell lines and tissue types. Based on *in vitro* experiments, the subcellular location of securin varies between several cell lines (Mu et al 2003) and may also vary in different phases of the cell cycle (Yu et al 2000, Stratford et al 2005). In normal oesophageal squamous epithelium securin has been described nuclear and expressed primarily in the proliferating cell layer, whereas the location changed into cytoplasmic in squamous cell carcinomas (Ito et al 2008, Zhang et al 2014). Expression in both cytoplasm and nucleus has been reported in other malignancies e.g. gliomas (Salehi et al 2013) and gastric carcinomas (Xu et al 2016).

According to some reports, however, securin is expressed as cytoplasmic both in benign conditions, such as pituitary adenomas (Tena-Suck et al 2008), as well as in malignancy, e.g. gastric (Wen et al 2004), prostatic (Zhu et al 2006) and hepatocellular carcinomas.

In gliomas, the subcellular location of securin has been discussed in association with the outcome of the disease, since predominantly nuclear expression has been observed in high grade tumours as opposed to cytoplasmic expression in low grade gliomas (Salehi et al 2013).

2.3.2 PTTG1IP

2.3.2.1 PTTG1IP in normal cells

Pituitary tumour-transforming gene 1 interacting protein (PTTG1IP), also called PTTG1 binding factor (PBF) is a 180 amino acid sequence possessing an N-terminal signal peptide, a transmembrane domain, a bipartite nuclear localisation signal and a tyrosine-sorting signal (Chien and Pei 2000, Imruetaicharoenchoke et al 2017). PTTG1IP has been found to be ubiquitously expressed in a wide variety of human tissues such as bone marrow, lymph node, pancreas and thyroid (Chien and Pei 2000).

According to present understanding, the subcellular location of PTTG1IP is dependent on a nuclear location signal (NLS) in the C terminus and a region necessary for interaction with securin (Chien and Pei 2000). The NLS in also required for the translocation of PTTG1IP from the cytoplasm to the nucleus (Chien and Pei 2000). In normal circumstances, PTTG1IP is located in both the cytoplasm and the nucleus. In the absence of the NLS, PTTG1IP is found completely in the cytoplasm (Chien and Pei 2000).

The full function of PTTG1IP remains still relatively poorly understood. The main function of PTTG1IP appears to be the nuclear transport of securin (Chien and Pei 2000, Vlotides et al 2007). In thyroid cells PTTG1IP represses iodine uptake by transcriptional regulation (Boelaert et al 2007). It has thought to have tumour transforming potential (Stratford et al 2005) but this has been questioned as mouse models overexpressing PTTG1IP didn't routinely develop tumours (Read et al 2011)

2.3.2.2 PTTG1IP and cancer

Soon after its discovery, PTTG1IP was found to be tumorigenic in mice (Stratford et al 2005). More recently, it has been reported to promote cell growth in hepatocellular carcinoma cells (Li et al 2013) and increase the invasive properties in thyroid and breast cancer cells (Watkins et al 2016).

In normal breast tissue, PTTG1IP expression is either low or non-existent (Watkins et al 2010). It has been suggested that its expression is, at least in part, controlled by oestrogen (Watkins et al 2010). PTTG1IP has been found to be overexpressed in breast cancer (Watkins et al 2010), where it has also been shown to promote invasion (Watkins et al 2010, Watkins et al 2016). In addition to breast cancer, PTTG1IP has been found to be overexpressed in two other types of malignancies, thyroid (Stratford et al 2005, Read et al 2017) and colorectal carcinomas (Read et al 2016). In thyroid carcinomas, PTTG1IP overexpression has been associated with unfavourable outcome (Hsueh et al 2013, Read et al 2017) and tumour recurrence (Stratford et al 2005).

The reason for the oncogenic properties of PTTG1IP has eluded research. The logical explanation appears to lie in the post-translational modification as among the few detected mutations of the *PTTG1IP* gene, none are known to have oncogenic properties (Imruetaicharoenchoke et al 2017). Another promising explanation might involve the association between PTTG1IP and P53 reported in thyroid and colorectal carcinomas (Read et al 2014, Read et al 2016).

2.3.2.3 PTTG1IP subcellular location in cancer

Based on *in vitro* experiments, PTTG1IP in cancer cells is mainly found in the cytoplasm, co-localized with securin (Stratford et al 2005). Based on evidence from thyroid cancer, it has been suggested that PTTG1IP is expressed in both the nucleus and the cytoplasm (Stratford et al 2005). Also, in colorectal carcinoma mainly cytoplasmic expression has been reported (Read et al 2016). However, some researchers have also reported on primarily membranous expression of PTTG1IP (Hsueh et al 2013).

2.3.3 Separase

2.3.3.1 Separase in normal cells

Separase (also extra spindle poles-like 1, ESPL1) is one of the main proteins responsible for maintaining the chromosomal integrity of the cell throughout mitosis. The *ESPL1* gene is located in the 12q13.13 chromosomal region and encodes the protein separase, which, in humans, is expressed in several isoforms. Separase is a cysteine protease and an endopeptidase with the main function to monitor chromosomal fidelity at metaphase-anaphase transition (Nasmyth and Haering 2009).

As the activation of separase in late metaphase triggers the cleavage and inactivation of the Scc1/Rad21 subunit of cohesin and the progression to anaphase, it must be accurately controlled to prevent a premature onset of anaphase. To maintain this control, there are several different mechanisms, independent of each other. The best-known regulator of separase is securin, as outlined in Chapter 2.3.3. Binding to securin inhibits the activity of separase during most of the cell cycle but when securin is degraded by the APC/C at the onset of anaphase, separase is activated and released (Shirayama et al 1999, Uhlmann 2003). Separase may also be inactivated via phosphorylation by cyclin B-dependent Cdk1 (Ciosk et al 1998, Gorr et al 2005). There is also research suggesting a still relatively poorly understood autocleavage mechanism may partake in this tight regulatory process (Zou et al 2002, Waizenegger et al 2002). However, it has been suggested that a low level of separase activity is maintained during the whole cell cycle (Kumar 2017) and that both securin and Cdk1 also have separase activating properties in addition to their inactivating functions (Hellmuth et al 2015). The activated separase at anaphase cleaves the cohesin binding the sister chromatids together, but only at the centromeres (Waizenegger et al 2000).

At interphase, separase is located in the cytoplasm. The relatively large size of the protein and the lack of nuclear localization signal prevent the transition through the nuclear membrane. In addition, separase contains a nuclear export signal, further increasing the restriction to the cytoplasm. Thus, only the destruction of the nuclear membrane during the prometaphase allows the separase to gain access to the nuclear proteins, i.e. cohesin (Zhang and Pati 2017).

In addition to functioning at the metaphase-anaphase transition, it has been suggested that separase participates in the cell cycle in spindle assembly, centrosome cycle, DNA damage repair and membrane trafficking (Zhang and Pati 2017, Hellmuth et al 2018). Based on experiments resulting in siRNA-induced depletion of ESPL1, separase has also been suggested with a conserved role in telomere protection (Cipressa et al 2016). Based on studies in healthy individuals, genetic

variations in *ESPL1* have been suggested to induce formation of chromosomal aberrations in the diving cell (Försti et al 2016). Abnormal function of separase halting the continuation of the cell cycle into anaphase has been reported to predispose the cell for premature separation of chromatids, lagging chromosomes and anaphase bridges, known indicators of chromosomal instability and aneuploidy (Zhang and Pati 2017). Consequently, ESPL1 has been associated with chromosomal instability (Fridlyand et al 2006, Ignacio Pérez de Castro et al 2007), single nucleotide pleomorphism (Brendle et al 2009) and included in a gene expression signature of aneuploidy in several human malignancies (Carter et al 2006).

2.3.3.2 Separase in cancer

Analyses of gene expression and comparative genomic hybridization have suggested the association of separase overexpression with loss of tumour suppressor genes *P53* and *Rb* (Pati 2008). In addition, response elements for oestrogen and progesterone have been detected in the *ESPL1* promoter region and separase overexpression has been associated with mammary tumorigenesis (Zhang et al 2008, Mukherjee et al 2014). More recently, Zhang and co-workers have identified Sepin-1, an inhibitor of separase, which has been shown to inhibit the growth of ER-positive breast cancer cell lines and breast cancer xenografts in mice and, in most cases, reflect the expression levels of separase in both cancer cell lines and in tumours (Zhang et al 2014). In summary, separase, when overexpressed acts as an oncogene with important roles in the malignant progression, and inhibition of separase constitutes a promising strategy for treating aneuploid cancers.

Previously, separase has been shown overexpressed in a wide range of human malignancies, including carcinomas of breast, prostate, and bone (Meyer et al 2009, Zhang and Pati 2017) and gliomas (Mukherjee et al 2014). Based on gene and miRNA expressions, separase has been associated with the pathogenesis and prognosis of endometrial and gastric carcinomas. Even though separase is often overexpressed on protein level in malignancies, the mutation frequency is relatively low (Zhang and Pati 2017)

In breast cancer, more than half of the tumours have been reported to be significantly overexpressed by separase as compared to the matched normal tissues (Zhang et al 2008, Zhang and Pati 2017). Particularly, the role of separase has been demonstrated in ER-dependent breast carcinomas based on experiments where carcinomas corresponding to luminal B tumours were developed in transgenic mice overexpressing the *Espl1* gene (Mukherjee et al 2014). The role of *ESPL1* as a driver oncogene in luminal B breast carcinomas has been supported based on profiling miRNA overexpression (Cornen et al 2014). Associations with clinic-pathological features of breast cancer have shown that separase overexpression in an independent

predictor of aggressive course of disease and poor survival in breast carcinomas, particularly in the luminal B subtype (Mukherjee et al 2014, Gurvits et al 2017).

2.3.3.3 The subcellular location of separase in cancer

Previous literature concerning the subcellular location of separase in cancer is sparse and partly contradictory. Also, it is not completely understood how overexpression and subcellular location of separase are interrelated or contribute to the formation and the clinical behaviour of the tumour.

The major part of the literature suggests that in tumour cells high separase expression is constitutively nuclear regardless of the proliferative status of the cells (Meyer et al 2009). On the other hand, separase has been described as a cytoplasmic protein, which is segregated from the nucleus during interphase by the nuclear envelope (Sun et al 2006). In addition, other expression patterns have been described and speculated to reflect the specific roles of separase in oncogenesis. Previously the role of nuclear localization of separase has been elaborated in glioblastomas, where separase was observed in both the nucleus and the cytoplasm, while nuclear expression indicated a higher incidence of relapse and disease mortality (Mukherjee et al 2014). Instead, aggressive clinicopathological features and reduced survival has been associated with cytoplasmic localization of separase in breast carcinomas, whereas non-neoplastic breast luminal epithelium showed nuclear expression of separase.

In previous literature, several explanations for the subcellular location of separase has been speculated, the most obvious being that the intensive overexpression of separase may overwhelm the normal mechanism of separase expression, the export of separase from the nucleus of proliferating tumour cells may be hampered of nuclear retention of separase results in premature removal of the sister chromatids (Nagao et al 2004, McAleenan et al 2013).

2.3.4 SA2

2.3.4.1 SA2 in normal cells

Cohesin is a multiunit protein complex responsible for the cohesion of the sister chromatids during metaphase assuring their proper alignment as presented previously in Chapter 2.3.3. In somatic cells, the human cohesin consists of four different subunits SMC1, SMC2, RAD21 and either SA1/STAG1 or SA2/STAG2 subunit. One cohesin complex is associated with either a SA1 or SA2 subunit, so that SA1 subunit is more likely associated with centromere and chromosome arms while a cohesin complex containing a SA2 subunit is mainly located at the telomeres

(Canudas and Smith 2009). These four proteins form a ring-like structure encasing the sister chromatids immediately after cytokinesis (Brooker and Berkowitz 2014). At interphase SA2 is located in the nucleus (Losada et al 1998) where is has been transported from the cytoplasm. This transportation is facilitated by a nuclear localization signal at the C-terminal fragment of the protein (Tarnowski et al 2015). At prophase, most of the cohesin complexes at chromosome arms are disassociated by phosphorylation of the SA subunits (Hauf et al 2005). At the centromeres cohesin is removed more slowly as the SA2 subunit protected from the hyperphosphorylation by shugoshins (McGuinness et al 2005). At the metaphase-anaphase transition the rest of the complexes are actively removed by separase activated by the APC/C cleaving the RAD21 subunit (Morales and Losada 2018), independent of the phosphorylation status (Hauf et al 2005). It is known that SA2 is dependent on RAD21 but not the other way around and depleting RAD21 from the cells destabilizes the SA2 (Vass et al 2003). Depleting SA2 from a cell does not lead to premature separation of sister chromatids whereas depleting RAD21 does (Vass et al 2003).

In addition to the role in sister chromatid cohesion, cohesin also functions in DNA damage repair at the S/G2 phase and the SA2 subunit – not SA1 – is responsible for the recruiting cohesin to the damage sites (Kong et al 2014). In DNA repair, some reports indicate that depleting SA2 from a cell increased the amount of SA1 in the cell (Kong et al 2014). This observation suggests a possible compensatory mechanism between SA1 and SA2 although this finding could not be confirmed by others (Solomon et al 2011). It has also been suggested that SA2 might also have a function as a transcriptional co-activator (Lara-Pezzi et al 2004).

In contrast to most cell cycle proteins, SA2 immunohistochemical expression can be found in most non-neoplastic tissues (Solomon et al 2011), including stromal fibroblasts.

2.3.4.2 SA2 in cancer

The loss of SA2 expression has been reported in several cancer cell lines such as glioblastoma (Solomon et al 2011), Ewing sarcoma (Solomon et al 2011, Tirode et al 2014, Crompton et al 2014, Brohl et al 2014) and malignant melanoma (Solomon et al 2011). Loss of expression has also been reported in a minority urothelial and pancreatic adenocarcinoma (Solomon et al 2013, Evers et al 2014) and the majority of gastric, colorectal and prostate carcinomas (Kim et al 2012). In urothelial carcinoma, the loss of expression was associated with a truncating mutation (Solomon et al 2013).

In prognostic evaluations, the loss of SA2 expression has been associated with increased disease mortality in invasive urothelial carcinomas (Solomon et al 2013).

Instead, in pancreatic carcinomas SA2 expression has been associated with higher survival rates, whereas the loss of expression predicted positive response to adjuvant chemotherapy (Evers et al 2014).

In literature, several somatic genetic mutations are proposed leading to the loss of SA2 expression in cancer cells (Solomon et al 2011, Taylor et al 2014). In some studies, these mutations have been associated with chromosomal instability and aneuploidy (Solomon et al 2011) while most studies have not confirmed this finding (Balbas-Martinez et al 2013). Interestingly, in glioblastoma cells correcting these mutations was observed to lead to regained expression of SA2 and rescue of chromosomal stability (Solomon et al 2011) but this result could not be repeated in urothelial cells (Solomon et al 2013).

At present no literature on the expression on the prognostic role of SA2 in breast cancer is available. However, the SA2 coding gene *STAG2* is included in some gene sets proposed as possible prognostic markers in breast cancer (Chang et al 2016).

2.3.4.3 The subcellular location of SA2 in cancer

As the amount of data on the expression of SA2 in malignancy is limited, the number of reports on the subcellular location of SA2 is also low. However, in the available publications, the expression of SA2 is reported to be nuclear as evidenced in Ewing sarcoma (Brohl et al 2014), urothelial carcinoma (Solomon et al 2013) and pancreatic adenocarcinoma (Evers et al 2014). The expression has also been found to be nuclear in the normal tissues such as the fibroblasts surrounding the tumour cells (Solomon et al 2013, Brohl et al 2014)

3 Aims

This study concentrates on expressions of specific regulatory proteins of the metaphase-anaphase transition of the cell division, and their potential value in predicting the prognosis of breast carcinoma patients.

The specific aims of this theses are:

1. To demonstrate the protein expressions of selected regulators of meta-phase-anaphase transition, Securin, PTTG1IP, separase and SA2 in breast cancer (I–IV)
2. To demonstrate the prognostic impact of securin, PTTG1IP, separase and SA2 in breast cancer (I–IV)
3. To evaluate the relevance of securin, PTTG1IP, separase and SA2 in relation to clinical prognosticators in breast cancer (I–IV)

4 Materials and Methods

4.1 Patient and tissue materials

4.1.1 Patients (I–IV)

The material consists of a total of 1135 women diagnosed and treated for invasive breast carcinoma (Table 5). The first cohort (n=781) was treated in Central Hospital of Central Finland, Jyväskylä, Finland, in 1987–1997, representing the era of national breast cancer screening. The second cohort was collected in association with Auria Biobank, Turku, Finland, and consisted of 354 women with invasive breast carcinoma treated between the years 2005 and 2015 in Turku University Hospital, Turku, Finland. The patient cases in both cohorts were non-consecutive.

All patients were treated with surgical resection or a mastectomy with sentinel lymph node biopsy and/or axillary lymph node evacuation when necessary. After surgery the patients received radiation therapy or adjuvant treatment with anti-estrogenic or cytostatic drugs based on the international guidelines for breast cancer treatment at the time of the diagnosis. All patients who had received neoadjuvant treatment were excluded from the material. Complete clinical data and follow-up data was collected from pathology reports and patient files. The patients were allocated into subgroups of the intrinsic classification according to the recommendations of the of the 12th St Gallen International Breast Cancer Conference Expert Panel (Goldhirsch et al 2011).

The maximum follow-up period in the material was 22 years and 6 months (mean 10 years). Causes of death were obtained from autopsy reports, death certificates and from the Finnish Cancer Registry.

Table 5 Summary of clinico-pathological parameters of the study I–IV).

	Studies					
	I	II	III		IV	
Total amount of patients	445	447	401	96	781	354
Mean follow-up time (range) (years)	10.0 (0.02-20.3)	10.0 (0.02-20.3)	10.0 (0.02-22.5)	5.1 (0.08-11.7)	12.4 (0.02-22.7)	9.4 (0.08-17.8)
Mean age at dg (range) (years)	61 (28-95)	58 (39-78)	56 (39-78)	62 (32-93)	61 (28-95)	60 (39-78)
Axillary lymph node positive n (%)	222 (50)	228 (51)	187 (46)	33 (35)	351 (45)	99 (29)
Mean tumour size (range) (cm)	2.4 (0.2-10.0)	2.4 (0.2-10.0)	2.4 (0.2-10.0)	2.7 (0.8-18.0)	2.3 (0.2-16.0)	2.4 (0.2-18.0)
Breast cancer deaths n (%)	142 (32)	152 (32)	141 (35)	21 (22)	234 (30)	57 (22)
Histological type n (%)						
Infiltrating ductal	356 (80)	358 (80)	332 (82)	96 (100)	589 (75)	354 (100)
Special type	89 (20)	89 (20)	72 (18)	0	192 (25)	0
Intrinsic subtype n (%)						
Luminal	343 (77)	154 (67)	281 (69)	0	528 (67)	208 (59)
Her2-amplified	31 (7)	37 (16)	51 (15)	0	145 (19)	0
Triple-negative	71 (16)	39 (17)	47 (17)	96 (100)	108 (14)	146 (41)

4.1.2 Tissue materials (I–IV)

The breast carcinoma specimen in were prepared according to the standard histology practice i.e. fixed in buffered formalin (pH 7.0) and embedded in paraffin. Both materials were collected in tissue microarrays (TMAs). This was performed first, by identifying in H&E staining two representative cancer cell areas. Next, the paraffin blocks were punched in these areas to obtain two tissue cores from each tumour. The diameter of the tissue cores was 0.6 mm – 1.5 mm.

4.1.3 Ethical considerations

The research was approved by the Regional Ethics Review Boards of Turku University Hospital and Auria Biobank, Turku Finland, Central Hospital of Central Finland, Jyväskylä, Finland and Finnish Cancer Registry, Cancer Society of Finland, Helsinki, Finland (permit numbers 6/2002, AB15-9859 and TK-53-716-16). All research procedures involving human participants were performed in accordance with the ethical standards of the World Medical Associations code of ethics (https://www.wma.net/policies-post/wma-international-code-of-medical-ethics/) and the 1964 Helsinki declaration and its later amendments (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/).

4.2 Histological methods

4.2.1 Immunohistochemistry (IHC) (I–IV)

The primary antibodies and their respective procedures of antigen retrieval and detection for identification of securin, PTTG1IP, separase and SA2 are summarized in Table 6.

Table 6 Details of IHC for securin, PTTG1IP, separase, SA2, ER, PR, HER2 and Ki-67.

	Origin	Clone	Source	Dilution	Antigen retrieval	Incubation
Securin	mouse	DCS-280	Abcam ab3305	1:100	Microwave	1h RT
PTTG1IP	rabbit	polyclonal	Abcam ab128040	1:500	on platform	20min 37°
Separase	mouse	6H6	Novus biologicals H00009700-M01	1:300	Microwave	1h RT
SA2	mouse	3C6	Abcam ab4463	1:500	sCC1	32min 37°
ER	rabbit	SP1	Roche	RTU	sCC1	24min 37°
PR	rabbit	1E2	Roche	RTU	sCC1	32min 37°
HER2	rabbit	4B5	Roche	RTU	sCC1	24min 37°
Ki-67	rabbit	30-9	Roche	RTU	sCC1	12min 37°

sCC1= standard Cell Conditioning 1 RTU=ready to use RT= room temperature

In short, IHC to detect securin and separase applied Lab Vision Autostainer 480 (Thermo-Fisher Scientific, Fremont, CA, USA) and detection using PowerVision+polymer kit, according to the manufacturer's protocol (DPVB+110HRP Immunovision Technologies, Vision Biosystems, Norwell, MA, USA), with diaminobenzidine as chromogen. SA2 applied Benchmark XT (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) and detection with ultraView Universal DAB Detection Kit (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA). PTTG1IP-IHC applied Discovery XT (Roche Diagnostics /Ventana Medical Systems, Tucson, AZ, USA) with OmniMap HRP and Chromomap DAB Kit (Roche Diagnostics /Ventana Medical Systems, Tucson, AZ, USA) for detection.

4.2.1.1 Interpretation of IHC (I–IV)

The IHC expressions of securin, PTTG1IP, separase and SA2 were registered as the percentage of positive cells analysed from sets of 100 tumour cells. In minimum 100 and in maximum three sets of 100 cells were evaluated. For securin and separase, also the subcellular location of staining was evaluated and registered as predominantly nuclear or cytoplasmic, or the combination of both (III–IV). Only tissue cores showing more than 100 cells were included in the studies. Control materials comprised whole tissue sections obtained from surgical reductions of normal breast and TMA cores representing benign breast tissue outside the tumour areas.

4.2.2 Detection of clinicopathological prognostic parameters (I–IV)

The primary antibodies and applied IHC procedures used in the antigen retrieval and detection ER, PR, HER2 and Ki-67 are summarized in Table 6. The IHC for detecting ER, PR, Ki-67 and HER2 was performed on Benchmark XT (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) and the signals were detected ultraView Universal DAB Detection Kit (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA). The interpretation of ER, PR and Ki-67 IHC was performed according to generally accepted international guidelines (Hammond et al 2010). On the bases of HER2-ICH (intensity score 2+ or 3+) cases were selected for gene amplification verification by *in situ* hybridization (ISH) (Wolff et al 2013).

HER2/Chr17 double ISH were performed using Benchmark XT (Roche Diagnostics/ Ventana Medical Systems, Tucson, AZ, USA), the *HER2* DNA and the Inform Chromosome 17 probe set, and the ultraView SISH detection kit to detect *HER2* (Roche/ Ventana) and the ultraView Alkaline Phosphatase Red ISH Detection Kit to detect *Chr17* (Roche/Ventana).

4.3 Statistical analysis (I–IV)

To begin with, the patients were categorized into subgroups exhibiting low versus high expression of the studied proteins. This was performed by selecting cutpoints based on, first, the observations of the IHC interpretations and, secondly, on statistical analyses involving the mean and median values of each parameter. Finally, the optimal cutpoint for each protein was verified based on univariate analyses where the chosen cutpoint produced the strongest prognostic value between breast cancer-specific survival vs. death in our material. Consequently, the cutpoint for securin was set at 10% immunopositive cells (< 10% vs. ≥ 10%) in agreement with previous literature (Talvinen et al 2008). The subcellular location of securin was categorized based on observed cytoplasmic expression of securin in the minority (< 50%) vs. the majority (≥ 50%) of the cells. Among the cases exhibiting the extreme ends of these patterns, a further separation was performed for the cases where < 10% vs. ≥ 90% of securin was observed in the cytoplasmic compartment of the cancer cell. For PTTG1IP the categorization between low vs. high expression was done at 10% of immunopositive cancer cells (< 10% vs. ≥ 10%). The extent of nuclear separase immunoexpression was registered as fraction of positive cancer cells (< 1% vs. ≥ 1%). For SA2, the categorization between low vs. high expression was done at 5% of immunopositive cancer cells (< 5% vs. ≥ 5%).

Intra-class correlation coefficients were used in reproducibility analyses reflecting the inter- and intra-observer consistencies between different observers and settings. In prognostic analyses, Kaplan-Meier estimates were performed to demonstrate the cumulative percentages of breast cancer specific mortality, and Cox's proportional hazard models were used to assess associations between protein expressions and disease outcome. Cox regression analysis was used to assess the prognostic associations adjusted for the established prognostic features, tumour size, axillary lymph node status, histological and intrinsic cancer type and histological grade.

Relations between the studied proteins and the established clinical prognosticators were qualified as hazard ratios (HRs) with 95% confidence intervals (CIs). Associations between the expressions of the proteins studied and the established clinical prognosticators were analysed by Fisher's exact and Wilcoxon rank sum tests, and the results were quantified as odd ratios (ORs) with 95% confidence intervals. P-values <0.05 were considered statistically significant. The validity of the proportional hazards assumptions was assessed both visually and numerically, and no marked deviation from assumptions was observed.

The computations were performed with SAS for Windows, Version 9.3 (I-III) and 9.4 (IV) (SAS Institute, Cary, NC, USA) Kaplan-Meier survival plots were generated using R 2.15.0.

5 Results

5.1 Expression of securin, PTTG1IP, separase and SA2 in breast cancer

5.1.1 Expression of securin (II)

In normal breast tissue, immunoreaction for securin was observed only in single cells (< 1%) of the luminal epithelium (Figure 5) (II). The expression was solely or primarily nuclear while cytoplasmic expression alone was not detected in benign breast specimen (II).

In breast carcinomas, securin immunoexpression was observed in average in 8.3% of malignant cells (II). In material allocated to intrinsic subgroups, a significantly higher fraction of immunopositivity was observed for TNBCs than for HER2-amplified or luminal carcinomas ($p<0.0001$) (Figure 4 in II).

Concerning the subcellular location of securin, we found that 38% of the cases showed cytoplasmic expression in the majority of the cancer cells. Most of these latter cases (64%) represented histological grade 3 tumours. Among the remaining cases in which cytoplasmic securin expression was observed in the minority of the cancer cells, histological grade 1 and 2 were overrepresented (38% and 51% of the cases, respectively) (II). Correspondingly, the average fraction of cytoplasmic securin positive cells varied according to the intrinsic subtypes from high in TNBCs to low in luminal carcinomas. In Cox regression analysis, even slight cytoplasmic expression (in <10% of securin positive cells) indicated increased probability for TNBC (OR 4.0, $p=0.002$, CI 1.6–9.5 %) (II).

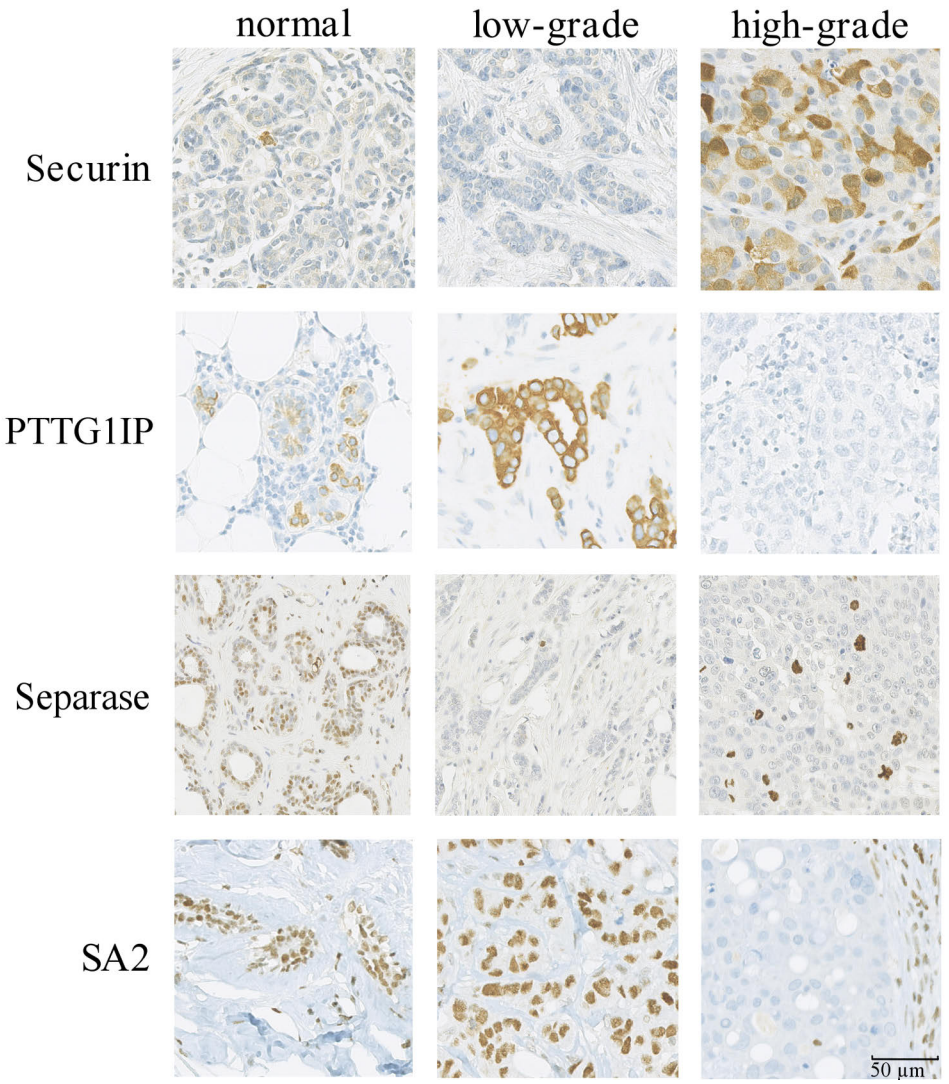

Figure 5 Photomicrographs showing immunoexpressions of securin, PTTG1IP, separase and SA2 in normal breast epithelium and in breast carcinomas representing low and high histological grade.

5.1.2 Expression of PTTG1IP (III)

In normal breast epithelium, single cells showed cytoplasmic PTTG1IP expression (Fig. 5) (III).

In breast carcinomas, PTTG1IP was observed as diffuse cytoplasmic staining expressed in an average of 74.3 % of the cases (III). The majority (56%) of carcinomas showed positivity in more than 10% of cancer cells (II). In material allocated into intrinsic subgroups, PTTG1IP was inversely associated with

aggressivity of the tumour as compared between luminal carcinomas vs. TNBCs ($p<0.001$) and between low vs high histological grade ($p<0.001$).

An association between expressions of securin and PTTG1IP was observed both morphologically and statistically among all carcinomas and in material divided into intrinsic subtypes. In statistical analyses, loss of PTTG1IP was related to the cytoplasmic location of securin ($p<0.0001$) (III). Also, morphologically, cancer cells exhibiting PTTG1IP-positivity showed nuclear securin expression while, in the absence of PTTG1IP cytoplasmic securin was observed (Figure 2 in III).

5.1.3 Expression of separase (IV)

In the normal breast, diffuse separase expression was observed in the nucleus and cytoplasm of epithelial cells, occasionally, also in the surrounding stromal cells (Fig. 5).

In breast carcinomas, separase immunopositivity was observed as nuclear positivity in mitotic cells (Fig. 5). Positivity for separase was observed in 31% of all breast carcinomas (I).

5.1.4 Expression of SA2 (I)

In normal breast specimen, SA2 was observed as a strong and uniform nuclear and cytoplasmic expression in both breast epithelium and stromal fibroblasts (Fig. 5).
In malignant cells, the pattern of SA2 expression was similar to benign breast epithelium and present in approximately one third (28%) of all breast carcinomas (I). Even in carcinomas with loss of SA2 expression in the malignant cells, positivity in stromal fibroblasts was still visible (I). An association between the reduced SA2 immunoexpression in cancer cells was observed in subgroups of different histological grades ($p=0.005$) and nodal status ($p=0.03$) (I). All morphologically identifiable mitotic figures were negative for SA2 in benign epithelium and stromal cells as well as in carcinoma cells (I).

5.2 Prognostic associations of securin, PTTG1IP, separase and SA2 in breast cancer

5.2.1 Prognostic associations of securin (I–IV)

Breast cancer survival was significantly associated with the fraction of securin expressing cells and with the subcellular location of the expression (Table 7) (II).

Table 7 Summary of prognostic impacts of securin, PTTG1IP, separase and SA2 as evaluated in univariate and multivariate analyses including nodal status, tumour size and histological grade as clinical features.

	univariate			multivariate		
	HR	P	CI	HR	p	CI
Securin, nuclear (II)	2.7	<0.0001	1.6-2.7	2.4	<0.0001	1.5-3.8
Securin, cytoplasmic (II)	1.6	0.003	1.1-2.4		ns.	
PTTG1IP (III)	1.5	0.02	0.9-2.7		ns.	
Separase (IV)	2.0	<0.0004	1.4-3.0	5.7*	0.002	1.9-17.2
SA2 (I)	1.6	0.02	1.1.2.5		ns.	

*luminal carcinomas only

High fraction of securin expressed cells (≥ 10% of cancer cells) indicated significant increase of breast cancer mortality in univariate analyses ($p<0.001$) (Table 8) (II). In multivariate analyses involving nodal status, tumour size and histological grade, securin showed independent prognostic impact (HR 2.4, $p<0.0001$, CI 1.5–3.8) (Table 3 in II). The intensity of securin immunoexpression did not have any statistical association with survival or with the clinical prognosticators of breast cancer (I).

Subcellular localization of securin was also found to predict outcome of breast cancer. Risk of disease-specific mortality was increased 1.6-fold among patients exhibiting securin expression predominantly (in ≥ 90% of the securin-expressing cancer cells) in the cytoplasm of the cancer cells ($p=0.003$, CI 1.1–2.4) (Figure 7) (II). When comparing the extreme ends of cytoplasmic securin expression (< 10% vs. ≥ 90% of the carcinoma cells showing cytoplasmic expression), the majority of the patients (75% quartile) with sparse cytoplasmic expression were alive after 11.2 years after diagnosis. Instead, among tumours with high expression of cytoplasmic securin, the patients' expected survival time was only 4.2 years after diagnosis (I). Cytoplasmic securin expression was also found to be associated with breast cancer mortality among in the patient subgroups divided according to nodal status, tumour size and histological and intrinsic classification but these associations were not statistically significant (I).

In multivariate analyses, the cytoplasmic expression of securin analysed together with nodal status, tumour size, histological grade and intrinsic classification failed to show statistical significance ($p=0.06$) (I).

5.2.2 Prognostic associations of PTTG1IP (III)

In univariate analyses, lack of PTTG1IP immunoexpression predicted a 1.5-fold increased risk of breast cancer death ($p=0.02$) (Table 7) (III). Survival curves of Kaplan-Meier analysis (Figure 6) revealed that in the majority (75%) of the patients with PTTG1IP-expressing carcinomas survived 11.4 years while the majority of patients with carcinomas lacking PTTG1IP expression were alive only 6.4 years after diagnosis (III).

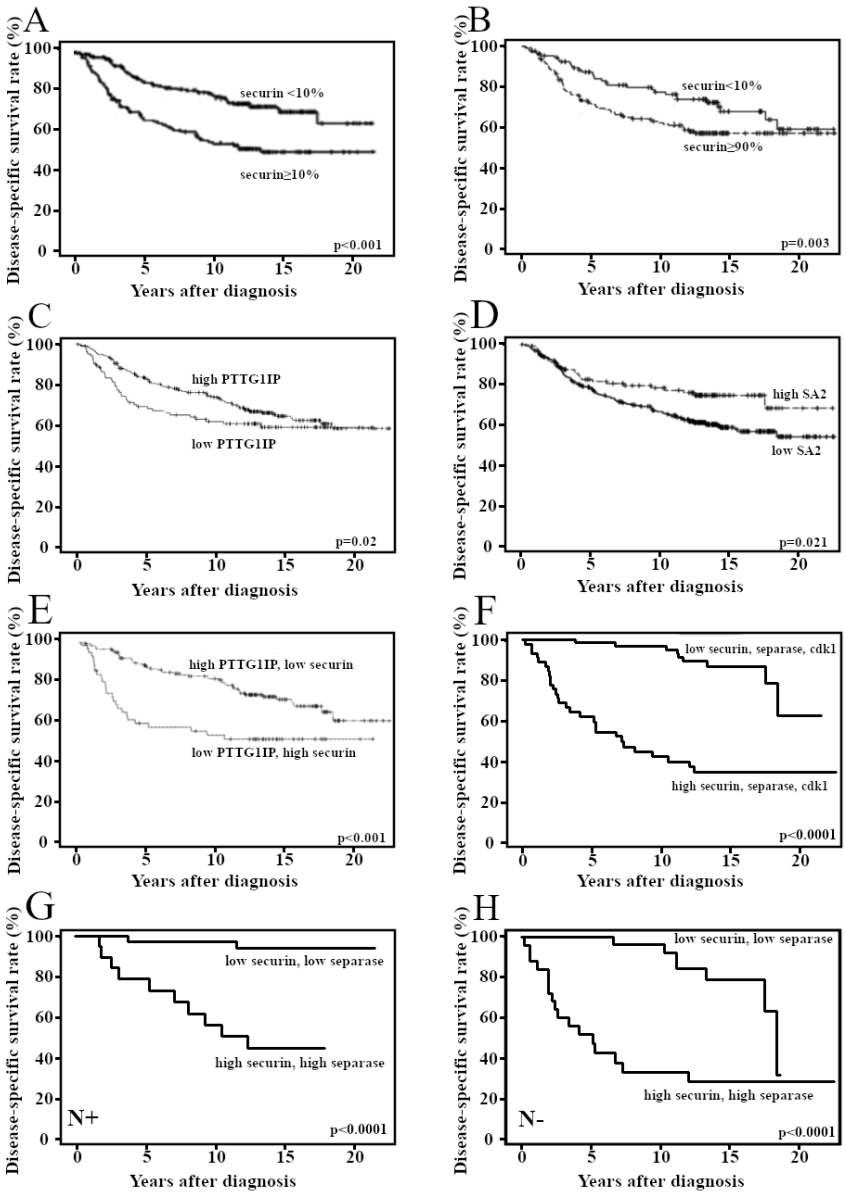

Figure 6 Kaplan-Meier curves representing breast cancer-specific survival determined according to the immunoexpression of securin, PTTG1IP, separase and SA2 alone (A-D) and in combinations (E-H based on a maximum of 22-year follow-up.

When evaluating the combined prognostic impact of PTTG1IP and securin, the subgroup of patients with tumours showing a low PTTG1IP expression and high securin expression was associated with a 2.3-fold ($p<0.001$, CI 1.6-5.4) increased

risk of breast cancer mortality as compared with patients exhibiting the combination of low securin and high PTTG1IP expression in their tumours (III).

In the multivariate setting, PTTG1IP expression did not show prognostic value in the whole material or patient subsets (III).

5.2.3 Prognostic associations of separase (IV)

In univariate analyses, high nuclear separase immunoexpression predicted doubled risk of breast cancer death ($p<0.0004$, CI 1.4–3.0). When tested in combination, high expression of separase and securin indicated significantly increased risk of breast cancer mortality ($p<0.0001$) (IV). Detailed quartile estimations of Kaplan–Meier analysis (Fig. 6) for individual patients suggested that the majority (75%) of patients with favourable combination of proteins (low expression) were alive 18.4 years after diagnosis, while unfavourable pattern of the proteins (high expression) suggested that one quarter (25 %) of the patients were dead of breast cancer after 2.5 years of diagnosis (IV). High separase also predicted breast cancer survival among luminal carcinomas (HR 5.7, $p=0.002$, CI 1.9–17.2) but not among TNBCs (IV).

5.2.4 Prognostic associations of SA2 (I)

In univariate analyses, immunoexpression of SA2 was significantly associated with breast cancer-specific mortality ($p=0.02$) (Table 8) (I). The lack of SA2 expression was associated with a 1.6-fold risk of breast cancer death (CI 1.1–2.5) (I). Concluding from Kaplan-Meier curves of survival analyses, the majority (75% quartile) of the patients with low SA2 expression were alive 6.0 years after the diagnosis, whereas the majority of the patients with high SA2 expression survived 17.6 years after the diagnosis (Fig. 7) (I). In addition, SA2 immunoexpression was significantly associated with metastatic disease of the axilla ($p=0.04$) and lack of *HER2* amplification ($p=0.03$) (I).

The combined prognostic impact of SA2 and securin was not statistically significant in the material (I).

In multivariate analysis involving SA2 with the established clinical prognosticators of breast cancer, SA2 did now show independent prognostic value (I).

5.2.5 The clinical relevance of securin, PTTG1IP, separase and SA2 in relation to the clinical parameters of breast cancer (IV)

In multivariate analyses, only securin and separase showed independent prognostic value when evaluated in combination with the established prognosticators of breast

cancer, axillary lymph node status, tumour size, histological grade and intrinsic classification (Table 8) (IV). Among all subtypes of carcinomas (n=781), the risk of breast cancer mortality was almost equally severe for patients with tumours exhibiting high securin immunoexpression and axillary lymph node metastasis (HR 2.5 and 2.6, respectively, p<0.001) (IV). In the current research, the independent prognostic value of separase was evaluated only among luminal breast carcinomas (HR 5.7, p=0.002, CI 1.9–17.2). Instead, PTTG1IP and SA2 did not show independent prognostic value in the material (IV).

Finally, the prognostic value of securin-related regulators of the metaphase-anaphase transition of the cell cycle were designed into multi-parametric models. In the development of the models, securin, separase, CDC20, PTTG1IP, SA2, Cdk1, and Cyclin B1 were involved and compared to the prognostic potential of the established prognosticators of breast cancer, axillary lymph node status, tumour size, histological grade, intrinsic classification, immunopositivity for ER, PR and Ki-67, and *HER2* amplification status (IV).

Table 8 Summary of multivariate analyses involving securin, PTTG1IP, separase and SA2 and the established prognosticators of breast cancer, axillary nodal status, tumour size, histological grade and intrinsic classification*. Also, results of the combination of securin, separase and cdk1 (model 1), and securin, separase and nodal status (model 2) are presented. Only statistically significant prognostic associations are shown. Results are presented for all breast carcinomas and for subgroups of axillary lymph node positive (N+) and negative (N-) patients, and luminal carcinomas.

Paper	Patients (n)	Features	HR	p-value	CI
II:	All (447)	securin	2.4	<0.001	1.5-3.8
		nodal status	2.8	<0.001	1.8-4.3
		tumour size	1.2	0.003	1.1-1.4
IV:	All (781)	model 1	8.4	<0.0001	3.4-20.7
		nodal status	4.3	<0.0001	2.6-7.0
	N+ (350)	model 1	6.5	0.0003	2.3-17.9
	N- (431)	model 1	19.5	0.006	2.3-163.8
	Luminal (208)	securin	1.1	0.02	1.0-1.2
		separase	5.7	0.002	1.9-17.2
		nodal status	4.9	0.003	1.7-13.7
		model 2	6.2	0.0006	3.2-82.6

The analysis has been performed on material divided into subgroups with favourable vs unfavourable outcome as follows: securin < 10% vs ≥ 10% of immunopositive cancer cells, nuclear separase < 1% vs ≥ 1% of immunopositive cancer cells, and tumour size < 2cm vs ≥ 2cm in diameter

Among all subtypes of breast carcinomas (n=781), the optimal model for detecting favourable outcome of disease involved securin, separase and cdk1. The unfavourable combination, i.e. high expression of all three markers (≥ 10% of cancer cells), separase (≥ 1% of cancer cells) and cdk1 (≥ 10% of cancer cells) indicated significantly increased risk of breast cancer death (HR 8.4, p<0.0001, CI3.4–20.7)

(IV). The Kaplan-Meier curves (Fig. 6) demonstrate the survival difference among all patients and N+ and N- patients (Fig 6). The survival curves indicate that the majority (>75%) of patients with tumours resulting in favourable combination of the model were alive 18.4 years after primary diagnosis while the unfavourable combination indicated 2.5-year survival for the remaining patients (25%) of the material (IV). Among the subgroup of N- patients, no cancer-related deaths were observed among patients exhibiting favourable combination of the model (IV). Instead, the unfavourable combination suggested cancer mortality for every fourth patient within 5.3 years from diagnosis (IV). Correspondingly, the majority of N+ patients with favourable and unfavourable combination were alive after 17.6 and 2.0 years from the primary diagnosis, respectively (IV). In multivariable analyses (Table 8), the designed model was observed with significant prognostic impact along with axillary lymph node status. Independent prognostic values for the model were observed also among N+ and N- patients (IV).

Verification of the designed model among luminal carcinomas and TNBCs indicated that the pair of securin and separase or the combination of securin, separase and axillary lymph node status comprised the most efficient prognostic impact among luminal breast carcinomas in predicting breast cancer death (HR 6.2, p 0.0006, CI 3.2–82.6) (IV). Instead in TNBCs, no statistically significant prognostic impact could be observed for any of the immunohistochemically studied biomarkers or clinico-pathological features (IV).

6 Discussion

In this study four cell cycle proteins, securin, PTTG1IP, separase and SA2, were characterized for their expression and assessed for their potential as biomarkers in predicting the prognosis of human breast cancer. The proteins were selected for analysis based on their known biological roles in metaphase-anaphase transition and on previous limited evidence of their prognostic potential in malignancy. Prognostic associations were derived from a maximum of 1135 breast cancer patients with complete clinical data and up to 22-year follow-up.

Breast cancer is known to be a heterogenous disease with varied morphological and molecular features, prognosis and response to treatment (Januškevičienė and Petrikaitė 2019). These features present the clinician with a major problem for diagnostic and curative therapy. Traditionally the prediction process of breast cancer has been attempted with clinical and histopathological markers, i.e. tumour size, lymph node status, metastasis, and the immunohistochemical detection of ER, PR, HER2 and proliferation (Lakhani et al. 2012). Lately, this classification has been attempted by genetic profiling and classifying the tumour to intrinsic classifications (van 't Veer et al 2002, Coates et al 2015). Consequently, there is still a significant need for additional tools and novel biomarkers to provide with more exact prognostic data on the probable course of disease and the optimal treatment for each individual patient.

6.1 Securin is overexpressed in human breast cancer (I–IV)

In the current research, normal breast ductal epithelium was practically negative in securin-IHC while 8.3% of invasive breast carcinomas showed securin-immunopositivity. Consistent with previous literature, among the positive cases, high extent of securin-expression was associated with aggressive clinical features, particularly with triple-negativity ($p<0.0001$) (II).

The oncogenic properties on securin were suggested soon after the discovery of the protein (Dominguez et al 1998). The overexpression of securin has been presented in several different types of malignancies such as breast cancer (Ogbagabriel et al 2005, Talvinen et al 2008), colorectal carcinoma (Heaney et al 2000,

Kim et al 2007, Zhou et al 2014), thyroid carcinoma (Boelaert et al 2001), prostate (Huang et al 2014), head and neck (Heikkinen et al 2016) and hepatocellular carcinoma (Cho-Rok et al 2006, Fujii et al 2006) while the ex-pression is very low in non-neoplastic tissues (Dominguez et al 1998, Zhang et al 1999). Several mechanisms for the overexpression of PTTG1 have been suggested, including genomic aberrations, somatic mutations and insufficient degradation (Bernal et al 2002).

In the breast, there is evidence on the essential role of securin in mammary gland morphogenesis. Particularly, the loss of securin expression has been seen to induce abnormal proliferation in ductal epithelial cells and spontaneous mammary gland tumorigenesis (Solbach et al 2004, Ogbagabriel et al 2005). Based on increased Pttg1 mRNA levels in experimental animals, securin has been speculated to be regulated in an oestrogen-dependent manner (Yin et al 2001).

Despite the known tumour-transforming properties and the commonly extensive overexpression of securin in cancer, the underlying mechanisms for its oncogenic properties are still largely unknown. Originally, the oncogenic properties of PTTG1 were attempted to be explained by stimulation of the basic fibroblast growth factor secretion causing angiogenesis and metastatic properties (Zhang et al 1999). The angiogenetic function has also been shown in vivo (Ishikawa et al 2001). However, securin has also been suggested to promote invasion and lymph node metastasis (Yan et al 2009), and function in epithelial to mesenchymal transition (Yoon et al 2012). There is also evidence that securin both binds p53 and modulates its transcriptional activity, providing an-other possible pathway for tumorigenesis (Yu et al 2000).

6.2 PTTG1IP regulates subcellular localization of securin in breast cancer (II, III)

In our studies, an association was observed between the expression patterns of securin and PTTG1IP. Statistically, depleted PTTG1IP predicted cytoplasmic expression of securin ($p<0.0001$). Also, morphologically in IHC and double-IF, PTTG1IP-positivity was associated with nuclear securin expression while PTTG1IP-negative tumours, most commonly representing TNBC, showed predominantly cytoplasmic securin. The current research suggests that the subcellular location of securin may is relevant for a proper function of the protein. The majority of breast carcinomas in the current study exhibited nuclear ex-pression of securin but among high-grade tumours and TNBCs predominantly cytoplasmic expression was overrepresented ($p=0.002$).

In the present research (II, III), PTTG1IP immunoexpression was observed only in single cells of normal luminal epithelium while the majority (74.3%) of breast carcinomas showed a diffuse cytoplasmic PTTG1IP immunoexpression. In breast

carcinomas, high immunoexpression of PTTG1IP was associated with favourable clinical and histological features while loss of PTTG1IP was observed in aggressive subgroups of breast carcinomas, such as triple-negative ($p<0.001$) and high histological grade ($p<0.001$).

In contrast to securin, PTTG1IP is found to be ubiquitously expressed in a wide variety of human tissues (Chien and Pei 2000). PTTG1IP has also been characterized as a tumorigenic protein (Stratford et al 2005) although this has later been questioned (Read et al 2011). It would appear to be capable of promoting cell growth (Li et al 2013). In normal breast tissue, PTTG1IP is either low or non-existent (Watkins et al 2010), being, at least in part, controlled by oestrogen (Watkins et al 2010). In breast cancer Pttg1IP is overexpressed (Watkins et al 2010) and has also been shown to promote invasion (Watkins et al 2010, Watkins et al 2016). Literature concerning the subcellular localization of securin in neoplasia is sparse although the cytoplasmic securin has been speculated in gastric (Xu et al 2016), testicular (Pierconti et al 2015), oesophageal (Ito et al 2008) and pancreatic carcinomas (Lin et al 2013), as well as brain tumours (Salehi et al 2013). According to literature, the subcellular localization of securin in non-neoplastic cells appears to be cell type-dependent (Mu et al 2003, Cai et al 2014). In normal luminal cells of the breast, securin is located in the nucleus of the cell. During the cell cycle, however, securin is degraded and then resynthesized in the cytoplasm. In neoplasia, the regulation of subcellular expression of securin is not settled. However, there is evidence that the translocation of the protein from the cytoplasm to the nucleus is mediated by a PTTG1IP (Chien and Pei 2000) although their precise roles remain undefined (Read et al 2017).

6.3 SA2 expression is lost in poorly differentiated breast carcinomas (II)

Among the core cohesion complex, RAD21 has emerged as the key biomarker the expression of which has been described for breast, ovarian, bladder and lung carcinomas (Rhodes et al 2004). Instead, very little has been published on the expression of SA2 in breast cancer and, therefore, SA2 was chosen as the target protein for investigating the regulation of cohesin-dependent sister chromatid separation in Study II. In the immunoanalysis, SA2 showed a distinct expression pattern. SA2 was found to be strongly and uniformly expressed in benign breast luminal epithelium and low-grade carcinomas while SA2 expression was absent in high-grade carcinomas ($p=0.02$).

The regulation of SA2 expression in breast cancer has not been settled although alterations in gene and protein expressions as well as targeting microRNAs have been speculated (Yan et al 2012).

6.4 Securin, PTTG1IP, separase and SA2 predict survival of breast cancer patients. (I–IV)

In the present material, the immunoexpression of each of the studied proteins was significantly associated with breast cancer-specific survival. High expression for both nuclear securin and separase predicted increased mortality whereas immunoexpressions for cytoplasmic securin, PTTG1IP and SA2 were adversely related to disease outcome. The prognostic impact of PTTG1IP and separase were intensified after combination with securin.

The prognostic associations of securin have been previously reported. Overexpression of securin has been demonstrated in breast carcinomas with aggressive morphology, metastatic spread and relapse (Solbach et al 2004, Ogbagabriel et al 2005). In clinical materials, securin overexpression has been associated with aggressive course of disease, nodal involvement and distal metastases, as well as resistance for radiation and cytotoxic therapy (Tong et al 2011, Grizzi et al 2013, Liao et al 2014). In addition, the expression of securin in the stroma surrounding breast cancer has been shown with prognostic impact (Bacac et al 2006). Moreover, the present patient material has previously been used to demonstrate the prognostic value of securin in breast cancer (Talvinen et al 2013, Gurvits et al 2017). Concerning other malignancies, several papers report on the association of securin overexpression with disease outcome in gastric, oesophageal, brain and endocrine tumours (Salehi et al 2013, Zhang et al 2014, Xu et al 2016, Romero Arenas et al 2018).

The prognostic value of separase has been previously acknowledged. However, the results are incomprehensive and partly contradictory. Previous literature reports on reduced and increased expression of separase in malignant disease (Kumar 2017). In humans, overexpression of separase expression has been observed in several malignancies, e.g. in breast and prostate carcinomas although the results are partly based on relatively small materials (Meyer et al 2009, Gurvits et al 2017). Separase overexpression has also been observed in association with aneuploidy DNA content in human breast cancer (Zhang et al 2008, Meyer et al 2009). In breast cancer, overexpression of separase has been observed as a characteristic of luminal B carcinomas (Finetti et al 2014). ESPL1 mutations causing loss of separase expression have been reported from renal and pulmonary carcinomas (Sak et al 2008, Gao et al 2019). Also, Separase mRNA levels have been associated with poor histological differentiation and mortality in breast carcinomas (Mukherjee et al 2014).

In the present material, the combined prognostic value of securin and separase was evidenced based on data from a total of 1135 breast cancer patients with complete clinical information and up to 22-year follow-up. Securin and separase combined into a model together with cdk1 showed equivalent prognostic value in predicting breast cancer mortality as axillary lymph node status. The results

suggested prognostic impact for our model also among node-positive and node-negative patients. Securin, separase and nodal status in combination predicted outcome of breast cancer also for luminal breast carcinomas, but not for TNBC. In breast cancer, multigene profiling assays (Xin et al 2017) have been established for clinical use to define breast cancer subtypes with individual molecular pathways predicting survival and/or treatment response. Simultaneously, various multiparametric prognostic models based on gene and protein expression are being developed for different clinical purposes, such as predicting disease recurrence (Lundberg et al 2017) or treatment response (Kurebayashi et al 2011). Recently, ESPL1 has been involved in a signature to predict the outcome of ER-positive breast cancer and response to neoadjuvant therapy in breast carcinomas (Buechler et al 2019a, Buechler et al 2019b). PTTG1 has also been combined into a prognostic signature predicting survival of papillary renal cell carcinoma (Gao et al 2019).

Concerning PTTG1IP, the evidence of prognostic impact is sparse and partly contradictory. However, the role of PTTG1IP in tumorigenesis through regulating p53 activity has been established in a number of reports (Read et al 2014, Read et al 2016). Most of the clinical prognostic trials involve thyroid cancer where PTTG1IP overexpression has been associated with poor prognosis (Hsueh et al 2013, Read et al 2017) and tumour recurrence (Stratford et al 2005). Also, in colorectal cancer, PTTG1IP has been associated with unfavourable outcome due to increased extramural vascular invasion, genetic instability and somatic TP53 mutations (Read et al 2016).

Research on the possible prognostic value of cohesin is sparse and mostly deals with SCC1/RAD21 subunits and not SA2. Only single papers report on the prognostic value of SA2 in cancer and the results are controversial. The loss of SA2 expression was detected in association with increased disease mortality in invasive urothelial carcinomas (Solomon et al 2013). Instead, in pancreatic carcinomas SA2 expression has been associated with higher survival rates, whereas the loss of expression predicted positive response to adjuvant chemotherapy (Evers et al 2014). Previously, SA2 has also been studied in acute myeloid leukaemia without detected significant prognostic associations (Thol et al 2014).

6.5 Benefits and limitations of the research (I–IV)

The present study is based on a relatively large patient cohort with a well-established clinical data and a long-term follow-up time reaching up to 22 years after diagnosis (mean 10 years). The cases originate from the era of national mammographic screening and, therefore, can be expected to be representative of the population. According to literature, the comparative number of histological grades vary in different breast cancer materials with grade I representing 11-38%, grade II 36-49%

and grade III 19-46% of the cases (Rakha et al 2010). In our material the distribution of the different grades was well in line with the literature being 27%, 50% and 23% for grades I to III, respectively. Similarly, the distribution of the different histological diagnoses and subgroups of intrinsic classification correspond to the prevalence presented for breast cancer in literature (Dai et al 2015).

The tissue samples of the patient material were collected from among the diagnostic specimen of clinical pathology practice. Due to the retrospective approach of the study, it may be speculated that the immunohistochemical detection of the studied proteins may have been compromised by the long archival time. Recently, the emerge of biobanks has enable conducting wide-scale research based on large collections of biological specimens and correlated clinical data (Paskal et al 2018).

For IHC, the specimens were collected into TMAs. TMAs are generally considered to improve the efficiency and cost-effectiveness of IHC. In addition, immunoevaluations in TMAs have been associated with high intra-and interobserver reproducibility (van Zwieten 2013). According to literature, immunohistochemistry on TMAs has been shown to have high concordance with whole tumour sections of the original tissue (Dixon et al 2015). Thus, results obtained from TMAs can be considered reliable, providing that the number of cases in the cohort is sufficient (Pinder et al 2013).

Intratumor heterogeneity is a common feature of malignancies and concerns particularly proliferation-related markers which, according to literature, show highest expression at the infiltrative front of tumours (Beliën et al 1999). In an attempt to manage the heterogeneity of the immunoexpressions, the TMAs on the present study were designed to include duplicate cores from every tumour. Based on literature and our own experience, however, the expression of the studied proteins did not considerable vary between different tumour compartments. Moreover, in order to ensure adequate representation of the studied protein in the tumour tissue, the interpretation of all immunostainings was started by examining whole tumour sections. In statistical analyses, the higher value obtained from the two cores was chosen for prognostic analyses further minimizing the influence of heterogeneous expression patterns.

Proteins are involved in virtually every biological phenomenon both in the normal and in neoplastic tissues. Genomic data is often insufficient in explaining biological processes while proteomic studies may provide understanding on disease processes, such as cancer, and identify means for therapeutic interventions. Since the last decades, the combination of IHC on TMAs has become an established method for detection and validation of novel biomarkers in cancer research (Hao et al 2004). IHC is a method of experimental as well as clinical practice and serves to correlate expression data to the morphological interpretation of tissues, and subcellular structures.

6.6 Securin, PTTG1IP, separase and SA2 have clinical relevance in breast cancer (I–IV)

Breast cancer is the most common malignancy and cause of cancer deaths among women in both Finland and worldwide. It has been estimated, that every 8th Finnish woman will be diagnosed with breast cancer during her lifespan (www.cancer.fi). Due to early detection and improvements in the diagnostic and therapeutic approach the current 5-year relative survival rate over 90 % (Finnish Cancer Registry 2019) Still, breast cancer is the most prevalent cancer in Finnish women and remains the major cause of cancer mortality. In addition, breast cancer and its treatment compose a considerable challenge for the quality of life among the increasing group of disease survivors. The high incidence of breast cancer together with being the leading cause of death all around the world has sparked the pursuit for detection of new and more efficient biomarkers for diagnostic, prognostic and predictive tools.

The current clinical prognostic and predictive parameters provide limited possibilities for prognostic classifications and categorization of carcinomas for treatments, leading to possible over- or undertreatment of individual patients. There are also limited means to predict the outcome of the treatments and guide the clinical decision-making progress. The high clinical relevance of breast cancer has resulted in the increasingly active publication. Still, only single biomarkers are recommended in clinical practice guidelines across (Fan 2013).

Proliferation has traditionally been used as a prognostic marker. The immunohistochemical evaluation of Ki-67 has traditionally been used as a tool for this evaluation. Also, the mitotic count in breast cancer cells is used in assessing the grade of the tumour. Metaphase-anaphase transition comprises one of the critical steps in the cell cycle and it is controlled by a complex network of regulators. The present study shows evidence that securin expression may serve as a strong and independent prognosticator of breast cancer outcome. According to the hallmarks of cancer, uncontrolled proliferation is one of the waypoints on the malignant progression of the cells (Hanahan and Weinberg 2011). With uncontrolled and uncoordinated cell proliferation the risk of checkpoint failure and chromosomal instability increases, leading to aneuploidy. Breast cancer is often known to harbour severe chromosomal instability, especially in triple-negative tumours. Interestingly, although several cell cycle proteins are included in gene panels predicting the behaviour of breast cancer, very few mutations have been found in the genes coding these proteins (Yuan et al 2006). The answer does not appear to be in single nucleotide pleomorphisms either (Brendle et al 2009). This leads to the conclusion that the answer lies somewhere in the epigenetic regulation, such as promoter methylation, of these proteins (Park et al 2007).

Recently, breast cancer treatment is experiencing a shift towards minimal surgery. As an example, lymphatic mapping and sentinel node investigations as

methods of evaluating local control of the disease and criteria for staging are currently being substituted by radiological evaluation of metastatic burden in the axilla (Nurudeen and Hunt 2018). Likewise, the increased use of neoadjuvant therapies results in more patients receiving systemic therapy before surgical removal of the tumour. These new developments in breast cancer therapy would greatly benefit from identification of biomarkers or biomarker signatures to individually predict the prognosis of the disease and the potential effectiveness of the treatments (Selli and Sims 2019). These developments emphasize the need for increasingly effective and progressively more targeted systemic therapies, also applying regulation of the cell cycle.

There is *in vivo* evidence, that manipulating the cell cycle checkpoint proteins provokes a massive chromosome loss and apoptosis, even in the setting of initially highly chromosomally instable cancer cells (Kops et al 2004). Only partially disturbing the chromosomal checkpoint resulted in minor chromosomal errors, but not cell lethality, and an increased sensitivity to the cytotoxic drug, taxol (Janssen et al 2009) or reversing cytotoxic drug resistance in the cells (Lee et al 2004). These results have opened the possibility of using the checkpoint proteins as possible druggable targets for cancer treatment.

Recently, securin has been suggested with potential as a therapeutic target for breast cancer (Grizzi et al 2013), particularly in case of ER-positive disease (Ghayad et al 2009). In addition, it has been suggested that depleting securin from the cells enhances radiosensitivity and induces senescence in the cancer cells, possibly due to disruption of the DNA double strand break repair function of securin (Chen et al 2010). Examining non-small cell lung carcinoma cells and nude mice Kakar et al (2006) targeted *PTTG1* mRNA by using small interfering RNA (siRNA) (Kakar and Malik 2006). This silencing resulted in down-regulation of *PTTG1* and reduced tumour growth *in vitro* and *in vivo*. These results have been repeated with hepatocellular carcinoma, both *in vitro* and *in vivo* (Cho-Rok et al 2006). In addition to siRNAS, similar results have been obtained using different methods. Mo et al. (2009) utilized the interaction of securin and PTTG1IP and created an engineered ubiquitin-protein ligase fusion protein with PTTG1IP, targeting overexpressed securin in both human cervical cancer line (HeLa cells) and activated monkey fibroblasts (COS-7) cells. The result was downregulation of securin and inhibition of cell growth (Mo et al 2009). Cho-Rok et el. (2006) targeted securin using an adenovirus-mediated transfer of siRNA, reducing tumour growth of hepatocellular carcinoma both *in vitro* and *in vivo* (Cho-Rok et al 2006). As clinical trials for adenovirus virotherapy are already taking place, this presents a promising treatment strategy.

In addition to securin, also separase has been suggested as a potential future molecular target for cancer therapy (Kumar 2017) other cell cycle proteins have also

been presented as possible predictive biomarkers or targets for cancer treatment. Ruppenthal et al (2018) have speculated that an increase in separase activity might be potential marker in identifying the malignant transformation of myelodysplastic syndrome to leukaemia (Ruppenthal et al 2018). Also, sepins - separase inhibitors – have been introduced as a potential novel cancer treatment, inhibiting tumour cell growth both *in vitro and in vivo.* Sepin-1 has been shown to inhibit the growth of human cancer cell lines and breast cancer xenograft tumours in mice by inhibiting cell proliferation and inducing apoptosis (Zhang et al 2014). The expression level of separase has been shown to predict the sensitivity to sepins in both cancer cell lines and tumours

Concerning SA2, it has also been shown that glioblastoma cells with harbouring mutations on the SA2 coding *STAG2* gene are more sensitive to PARP inhibitors (Bailey et al 2014). In melanoma cells loss of STAG2 function increased the resistance to BRAF inhibition (Che-hung Shen et al 2016). In pancreatic ductal adenocarcinoma is affected more by chemotherapy is more efficient if the expression of SA2 is low (Evers et al 2014).

Based on our results, cell cycle proteins, particularly securin and separase, could be used to predict the prognosis of breast cancer patients and possible used to modulate treatment decisions. However, further research is still required. In the future, there are still interesting research avenues such as the association of p53 with securin in breast cancer yet to revealed.

7 Conclusions

In this thesis, the immunohistochemical expression of and the subcellular location of securin, PTTG1IP, separase and SA2 has been evaluated in association with the prognosis of breast cancer.

1. The expression of securin, PTTG1IP, separase and SA2 in breast cancer differs from their expression in normal breast epithelium. In breast cancer, increased expression of securin and separase is associated with aggressive tumour morphology while the expression of PTTG1IP and SA2 is lost in poorly differentiated tumours. In high-grade breast carcinomas, the subcellular expression of securin shifts from the nuclear to cytoplasmic while PTTG1IP expression is simultaneous lost.
2. Securin, separase, PTTG1IP and SA2 predict the prognosis of breast cancer. The strongest prognosticators are securin and separase which predict at least doubled breast cancer mortality.
3. Among all breast carcinomas and luminal carcinoma, securin and separase alone and combined into prognostic models show independent prognostic value exceeded only by the impact of axillary lymph node status.

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"With magic you can turn a frog into a prince. With science you can turn a frog into a PhD and you still have the frog you started with"
–Terry Pratchett, The Science of Discworld–

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Heli Repo

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